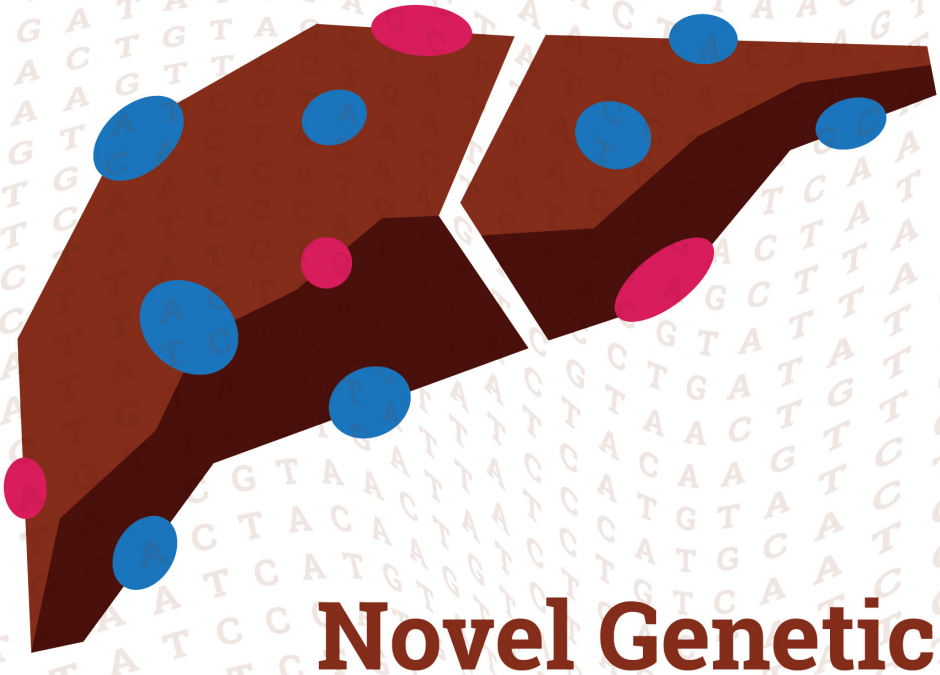

Novel Genetic Approaches in Polycystic Liver Disease

Wybrich R. Cnossen

NOVEL GENETIC APPROACHES IN POLYCYSTIC LIVER DISEASE

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

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GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

Hepatic and Renal Cysts

In general, cysts are fluid-filled, benign tumors. These structures correspond to cavities in organs. Cysts may be identified in multiple parts of the human body. For example, in the lung, pancreas, liver, kidney, spleen, bone and brain.[1-5] In the majority of cases, these clinical manifestations are not related to a syndrome or recognized as an inherited disorder. This is different for hepatic and renal cystogenesis.

Non-hereditary or acquired **hepatic cysts** occur in 2.5-4.7% of the general population and 11% of hospitalizations.[6] These simple cysts are variable in size on initial detection and may increase in size over time. They are often asymptomatic or detected as an incidental finding on abdominal imaging.

The **heredity** of hepatic cysts has been described since 1929.[7] Hepatic cysts may already be present as microscopic lesions at birth. Between birth and the third decade of life development of new hepatic cysts is rare, but may also be asymptomatic.[8] Small embryonic remnants are also called Von Meyenburg complexes. These small cysts usually remain silent during life in contrast to autosomal dominant polycystic liver disease (PCLD). The onset of symptomatic hepatic cysts may start around the age of 40 in PCLD. Although there is no evidence for genetic predisposition in woman, an increased frequency of hepatic cysts is demonstrated in female patients.[9]

The aspect of a **cyst** wall is smooth, transparent, avascular, yellowish/ bluish/ white in color (Figure 1). Cysts are formed by a thin layer of fibrous tissue lined by a single layer of cuboidal

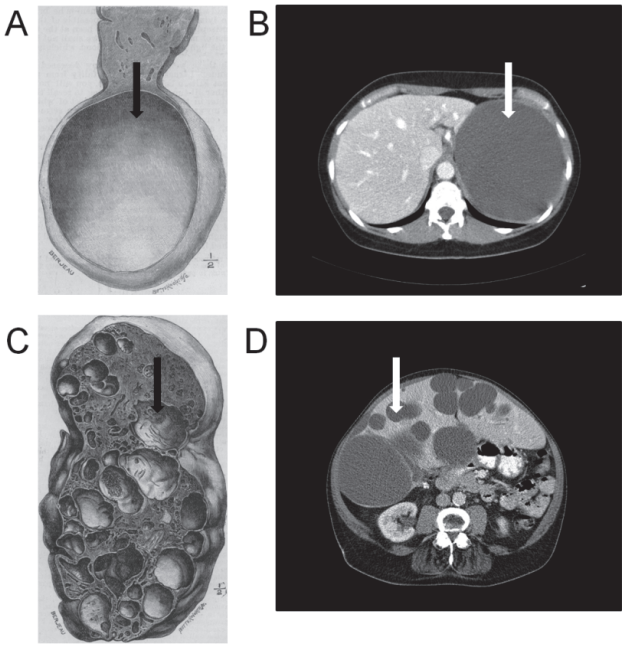

Figure 1. Simple hepatic cyst and polycystic liver. (A) Protruding hepatic cyst (black arrow) from the free border of the liver.[11] (B) Axial CT scan of a large hepatic cyst indicate by a white arrow. (C) Liver section presenting multiple large cavities. (D) Axial CT scan of a PCLD patient.

epithelia. Hepatic cysts may contain homogeneous transudate-like, clear or straw-coloured fluid of low viscosity.[10]

In the liver, cysts are the most common space-occupying structures. The size of hepatic cysts may vary from microscopically small to massively enlarged up to ~20 cm diameter.[12] PCLD patients may present over 20 hepatic cysts spread throughout the liver parenchyma.[13] Therefore, the major consequences of enlarging hepatic cysts are obstructive and mechanical **symptoms**. Frequent clinical signs of symptomatic polycystic livers consist of flank pain, abdominal discomfort, palpable mass or cyst-related complications.[14]

Hepatic cysts may co-occur with **renal cysts**. In PCLD, renal cysts are rarely seen and may be discrete lesions in the kidneys. Typically, few renal cysts are cortically located and distort the renal contour. The prevalence of renal cysts in the general population has been estimated to be 10.7-11.9%.[15, 16] Large cysts in both the liver and kidneys are more frequent in autosomal dominant polycystic kidney disease (ADPKD).

Ductal Plate Malformation

The morphogenesis of the **intrahepatic bile ducts** (IHBD) is established by a process of **ductal plate formation** after 7 weeks of gestation.[17] The ductal plate is recognized as the source of stem cells (hepatoblasts) for bile duct neogenesis and development. At different developmental stages hepatoblasts express cytokeratins (CK) that are intermediate filaments of the cytoskeleton. For example, CK8, CK18 and CK19 are expressed in early progenitor hepatoblasts.[18] Finally, normal IHBD express CK7 and CK19 as particular phenotypical markers which are absent in hepatocytes.[19, 20] Development of normal bile ducts and a classification of three pathogenic mechanisms for **ductal plate malformation** (DPM) are described in Figure 2.[21]

DPM can be seen as the basic component of congenital cystic diseases in IHBD and is caused by an excess of embryonic biliary structures with persistent ductal plate configuration. DPM compromises all abnormal gestational features that may occur during the bile duct development until the formation of mature IHBD. Disturbed epithelial-mesenchymal interactions in DPM

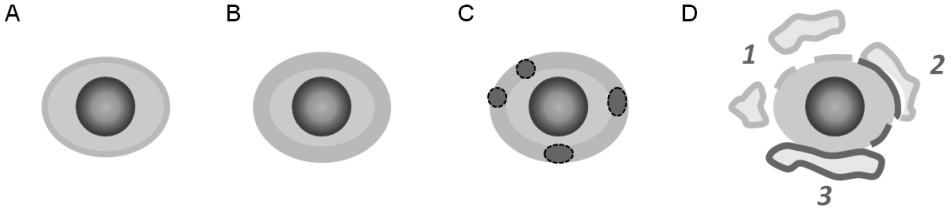

Figure 2. Concept of bile duct formation and ductal plate malformation at 8-12 weeks of human gestational age. This figure illustrates hepatoblast proliferation from a monolayer (A) to a double-layer around the portal vein (B). (C) Development of normal human bile ducts by focal dilatation between both layers from the 12th week of gestational age. (D) DPM and cyst formation may arise according to three mechanisms [21]: 1) abnormal hepatoblast differentiation, 2) failure hepatoblast maturation, 3) bile duct expansion with normal cell differentiation and maturation.

may cause ineffective remodeling of ductal plates, abnormal hepatoblast differentiation, and alterations in portal vein, bile duct configuration and cilia formation.[22]

DPM may occur in multiple intrahepatic and extrahepatic disorders such as extrahepatic biliary atresia, congenital hepatic fibrosis, autosomal recessive polycystic kidney disease, Caroli syndrome and Ivemark's syndrome.[23] In addition, DPM may occur in a spectrum of abnormalities at different levels of the biliary tree. For example microscopic, peripheral bile ducts in congenital hepatic fibrosis, and large bile ducts in Caroli syndrome. The isolated liver cysts in ADPKD probably represent DPM affecting the most biliary system.[24]

Moreover, in liver disease of ciliopathies DPM is the main underlying pathophysiological mechanism.[20] Ciliopathies describe a class of disorders with a dysfunctional cilium or cilium-anchored structures. In general, these diseases are inherited and present a broad phenotypic spectrum affecting multiple organs. ADPKD is classified as ciliopathy and also PCLD can be considered as an ciliopathy.[25]

Polycystic Liver Disease Phenotypes

Clinical Variation in PCLD

Polycystic liver disease (PLD) is a clinical condition in adult individuals affected with PCLD or ADPKD. In both diseases the phenotypical expression is heterogeneous. Clinical characterization of PCLD patients reported presence of asymptomatic renal cystogenesis in clinically diagnosed PCLD patients.[26, 27] Renal disease and progressive renal failure are frequently present in ADPKD patients. Genetically, PCLD and ADPKD are distinct diseases, however it is obvious that there is an overlap in phenotype between both disorders.[28]

A correlation between phenotype and genotype is not established in PCLD. Previous studies presented clinical variation in families with mutations in the *PRKCSH* gene.[29, 30] Asymptomatic individuals may have microscopically small or few hepatic cysts. Absence of hepatic cysts is related to a certain non-penetrance in PCLD.[13, 28, 30]

Variation in phenotype has not been ascribed to the causative gene mutation.[28, 29] Also the number of hepatic cysts may not be predicted from the mutated gene.[29] In several families mutations may occur due to founder effect in certain geographical regions of the Netherlands. In addition, a large number of singleton cases with a negative family history are identified. This observation of clinical variation suggests that the reported prevalence of PCLD is probably underestimated.

ADPKD Clinical Heterogeneity

First, an evident clinical heterogeneity was determined between ADPKD patients. Severely affected ADPKD patients were called polycystic kidney disease 1 patients because of linkage to the *PKD1* gene. These *PKD1* patients were clinically compared to non-*PKD1* patients. Important observations in non-*PKD1* carriers were the detection of fewer renal cysts at the time of diagnosis, less development of hypertension and diagnosis at an older age.[31]

APDKD is a monogenic renal disorder with a penetrance of renal cystogenesis of nearly ~100%. The consequences of a germline mutation in the *PKD1* or *PKD2* gene are clearly different. Identification of the gene and mutation type contributes to the prediction of renal disease severity. Although the cyst growth rates are similar in both *PKD1* and *PKD2* carriers, ADPKD patients

carrying a *PKD1* mutation develop earlier polycystic kidneys.[32] In addition, affected individuals with *PKD1* may progress 20 years earlier to end-stage renal disease compared to *PKD2*.[33]

There is an inter- and intrafamilial phenotypic variability in ADPKD. Regarding *PKD1* affected families clinical differences are poorly understood.[34] Involvement of modifier genes have been suggested to explain the early disease progression and severity in *PKD1* carriers.

In line with these findings, there are more mechanisms reported to be associated with a spectrum of polycystic kidney disease phenotype. Atypical mutations or unusual mode of inheritance were identified during the search for a putative *PKD3* locus. I will describe several aspects of these genetic investigations.

Genetic Mechanisms in ADPKD

1) Hypomorphic allele variants/ incomplete penetrance: Two clinically diagnosed autosomal recessive polycystic kidney disease (ARPKD) patients developed severe renal disease in utero. However the phenotype overlaps with early-onset ADPKD, the family history was negative. Molecular screening of both parents revealed hypomorphic *PKD1* variants. Presence of two hypomorphic *PKD1* variants *in trans* explained the ADPKD phenotype.[35] Therefore, the dosage of functional PKD1 protein may be critical for cyst development.[36]
2) Mosaicism: Exceptional cases describe the clinical relevance of mosaicism in transmission of the defect to offspring. This genetic mechanism may explain patients with early-onset of polycystic kidney disease or may be considered in case of excluding *PKD1* and *PKD2* variants for living kidney donor transplantation.[37-39]
3) Digenic model: A family with *PKD1* and *PKD2* gene mutations reported different onset of end-stage renal disease. The most severely affected individuals carried mutations in both ADPKD genes in contrast to single gene mutations. A threshold model was proposed to explain the pathophysiological mechanism for these digenic and monogenic mutation carriers.[40]
4) Modifier genes: In patients with perinatal or early-onset renal disease during childhood additional mutations were identified in *PKHD1* or *HNF1*β.[41] This study proposed that multiple genes are involved in severe polycystic kidney disease phenotypes.
5) *De novo* ADPKD cases: Patients with an apparent negative family history may be examples of *de novo* disease manifestation.[42] One study reported evidence for six *de novo* cases.
6) Association or overlap with other diseases: The *tuberous sclerosis complex* gene is adjacent to *PKD1* on chromosome 16. Recent studies have shown that genomic deletion of *PKD1* and *TSC2* may result in earlier and more severe renal disease in a small number of patients.[43]

Severe Polycystic Liver Disease

The definition of massive PLD may include several aspects such as number and size of hepatic cysts, distribution of cysts in both liver lobules and complications. Patients with severe PLD have extremely enlarged livers up to ~eight-fold in size. Disabling symptoms due to the mass-effect impair the physical condition and quality of life in patients.

Familial clustering of massive polycystic livers in PCLD or ADPKD is unknown. For example, the *PKD1* mutation position is strongly related to an increased risk for cerebral aneurysms in ADPKD. *PKD2* carriers are at risk for vascular complications.[44] Such evidence is unknown for polycystic livers which is the most common extra-renal manifestation.

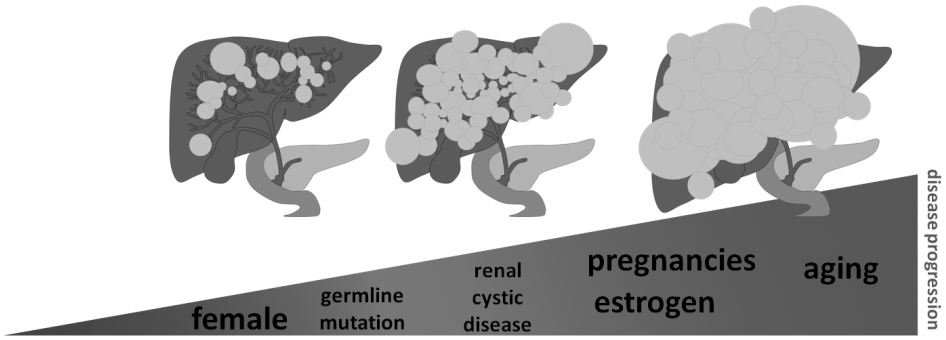

Figure 3. Determinants positively related to hepatic cystogenesis.

There are several aspects which may be related to progressive PLD (Figure 3). For example, the severity of hepatic cystogenesis in ADPKD is related to renal disease.[45] Therefore, the type of ADPKD gene mutation and renal disease progression are important factors indirectly associated with PLD.[45, 46]

Pregnancies and estrogen administration may be considered as hormonal factors or as environmental influences.[9] These factors are positively correlated with hepatic cyst enlargement.[47] Finally, increasing age is associated with cyst growth. The question remains if there are more environmental or organ-specific factors triggering massive hepatic cystogenesis.

Polycystic Liver Disease Genes

PKD1 and PKD2 associated with ADPKD

In the early 1990s, gene discoveries for ADPKD have been conducted by **positional cloning** (Figure 4). First genetic linkage studies mapped a major locus to chromosome 16 for ADPKD.[48] The region was more concisely determined to a 600-kb interval on 16p13.3.[49, 50] The European polycystic kidney disease consortium identified one splice site defect, two deletions and a *de novo* chromosome translocation in the ***PKD1*** (*PBP*) gene.[51] One deletion was also reported as a *de novo* event. These findings confirmed a causative role of *PKD1* mutations in ADPKD.

After initial linkage analysis, genetic heterogeneity of ADPKD was evident.[52] Concurrent linkage studies searched for a second ADPKD gene. Few years later, nonsense mutations were identified in the ***PKD2*** gene (chr.4q21) in three unrelated ADPKD families.[53]

Since conventional approaches excluded linkage to either the *PKD1* and *PKD2* locus in several ADPKD families, involvement of another locus was suggested.[54-57] These families presented ADPKD with mild renal phenotype or a severe associated extra-renal manifestation. A hypothetical ***PKD3*** gene might explain the clinical heterogeneity.[58]

In addition, a rodent model with polycystic kidneys and hepatic cysts unlinked to *PKD1* and *PKD2*, demonstrated linkage to a locus homologous to human chromosome 8q23-24.[59] Candidate genes in this region near the proenkephalin gene (*PENK* gene; chr.8q12.1) in rats were identified on human chromosome arms 6q and 9p.

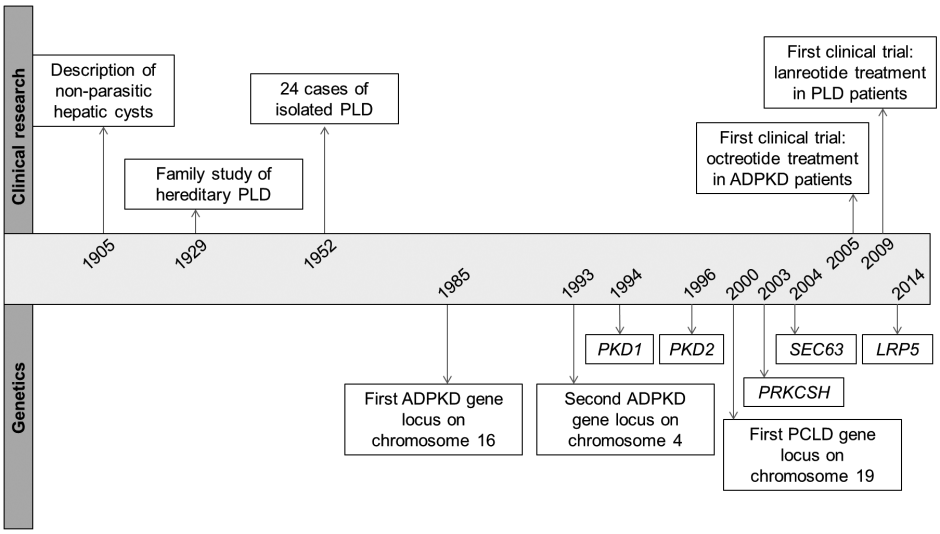

Figure 4. Timeline highlighting clinical studies and achievements in genetics in PLD.

More research groups reported unlinked ADPKD families with mild, but also severe phenotypes similar to *PKD1* carriers.[58, 60] For example, clinical follow-up of an unlinked ADPKD family with severe renal and systemic manifestations presented renal disease progression.[60, 61] This clinical heterogeneity observed in non-*PKD1*/*PKD2* families resulted in speculations about a role of **modifier genes**.[61]

Genetic Approaches in ADPKD

Rapid technological developments enabled improved detection of variants in the human genome. Repeated clinical and sequence analysis of previously reported unlinked ADPKD families from five different origins [54, 56-58, 61], did reveal one *PKD2* and three *PKD1* mutations.[62] Still, **direct Sanger sequencing** and **multiplex ligation probe-dependent amplification** (MLPA) of *PKD1* and *PKD2* identified no causative mutation in 11-13% of ADPKD families from the Consortium for Radiological Imaging Studies of PKD (CRISP) population.[43, 63] The same is true for another large ADPKD cohort where comprehensive mutation analysis showed 71 mutation-negative unrelated ADPKD patients (10.1%).[64] The clinical features of ADPKD families without *PKD1* and *PKD2* mutations are heterogeneous with a large proportion of mild phenotypes.[65] These findings point to another ADPKD locus as previously described.

Direct Sanger sequencing is the 'golden standard' for detection of point mutations and other small genetic variants, but complete sequencing of the *PKD1* gene remains difficult.[63] For example, larger genetic variants are missed. Therefore, mutation-negative ADPKD families may also be explained by undetectable variation or chromosomal rearrangements in both *PKD1* and *PKD2* genes.

Recent development of **high-throughput sequencing technologies** may give information about large DNA rearrangements, exonic and intronic variants with unpredicted consequences

on splicing, mosaicism or hypomorphic alleles. It is likely that these yet undetected types of variants may contribute to ADPKD.

The first application of **next-generation sequencing** (NGS) in ADPKD is a design for *PKD1* and *PKD2* **targeted re-sequencing**. Likely pathogenic deep intronic sequence variants, gene conversions and allelic drop-out were successfully called in 10% of previously mutation-negative ADPKD patients.[66]

PRKCSH and SEC63 associated with PCLD

PCLD and ADPKD were clinically defined as separated entities. After identification of both ADPKD genes, the clinical condition of isolated polycystic livers was also designated as a genetically distinct condition. Pathogenic variants in the *PKD1* and *PKD2* gene were excluded in PCLD patients by three groups.[67-69] In 2000 a locus on chromosome 19 was reported to be associated with PCLD in two large families.[13] **Positional cloning** for the three known loci for polycystic liver disease, 19p13.2, 16p13.3 (*PKD1*) and 4q21 (*PKD2*), in 8 Finnish families considered PCLD as a genetically heterogeneous disorder.[70]

Three years after the identification of locus 19p13.2-13.1, the first gene was simultaneously identified by two groups using linkage analysis.[30, 71] Both groups reported different pathogenic variants in the ***PRKCSH*** gene resulting in loss-of-function. Four extended and unrelated Dutch families revealed two splice site mutations.[30] In 6 out of 25 families unique splice site, nonsense and frameshift variants leading to a premature stop in the *PRKCSH* gene were identified.[71] In addition, linkage analysis in previously non-*PKD1*/*PKD2* families with a clinical diagnosis of PCLD [68-70] identified six pathogenic variants in the ***SEC63*** gene (chr.6q21).[72] These *SEC63* mutations included nonsense, frameshift and splice site variants, and a 3-base pair deletion. Until now, 25 *PRKCSH* mutations and 22 *SEC63* mutations have been identified by linkage mapping in combination with Sanger sequencing.[73]

The identification of both genes led to investigations of the **phenotype** in greater detail.[29, 74] PCLD is characterized by a clear autosomal dominant inheritance pattern. The **penetrance** has been estimated less than 100%.[13, 28, 71] The PCLD non-penetrance was already reported in two males (age 34 and 44) and one female (age 35) members of a Dutch family.[30] Although incomplete penetrance is rare, families affected with a *PRKCSH* or *SEC63* mutation with unaffected carriers have been reported.[29, 30, 74] Incomplete penetrance may easily be confused with variable disease expression. Here, the question is: what causes such a wide range of disease severity?

In addition, extensive *PRKCSH* and *SEC63* mutation analysis reported 78-84% PCLD families unlinked to both genes.[28, 29, 74] This suggests a high genetic heterogeneity for PCLD. Therefore, I hypothesize that at least one more **locus** is involved.[28] In addition, we question whether PCLD is a genetically heterogeneous monogenic disorder. Involvement of modifier genes may explain variable disease phenotype in patients with a similar *PRKCSH* or *SEC63* mutation.

Characteristics of Gene Products in PLD

Although the predominant ADPKD phenotype is polycystic kidneys, a ubiquitous polycystin tissue expression is in line with the multi-systemic manifestations in ADPKD.[53, 75] Likewise, both PCLD protein products, hepatocystin and Sec63p, have a **wide tissue expression** including the liver, kidney, pancreas, heart, brain and muscle.[30] These findings are perhaps surprising

as the PCLD phenotype is restricted to the liver. In addition, expression of the *PKHD1* protein product polyductin (fibrocystin) in ARPKD is only observed in the kidney, pancreas and at low levels in the liver.[76] This may explain why ARPKD patients are primarily affected with polycystic kidneys and CHF. It is questionable whether there is another, yet unknown, locus associated with PCLD with confined protein expression in the liver.

In the context of protein localization and function of hepatocystin and Sec63p, both are transmembrane proteins involved in glycoprotein folding and quality control.[77] A possible third PCLD protein product may be located in the **endoplasmic reticulum**. In addition, hepatocystin and glucosidase IIα are both required for the synthesis or transfer of glycans. Following examples of congenital glycosylation disorders such as CDG-1, variants were repeatedly identified on subunits from a multi-complex protein.[78, 79] It remains unclear whether protein components of hepatocystin and Sec63p may be functional involved in hepatic cystogenesis.[80]

Another hypothesis is that there exists an interaction network between PCLD and ADPKD genes.[81] This may explain the overlap in phenotype. Therefore, molecular pathways involved in ADPKD are also important in the search for unknown PLD genes.

Research Strategies and Techniques

The primary aim of PLD research is the development of treatment possibilities to improve patient's health in general. Current clinical research is focused on reduction of total liver volume and preservation of quality of life.[14, 82] There are a number of surgical and radiological interventions available that may be effective in selected cases.[83] In patients with advanced PLD, these procedures are less effective or are prone to complications. Hence, the need for new or improved medical treatment options. These PLD treatment possibilities are mainly studied in *in vivo* animal models and *in vitro* cell cultures.

Studies in PLD Animal Models

Extensive **pre-clinical studies** prior to drug administration are necessary to meet ethical issues and importantly as patient's safety. The beneficial effects of proposed medical treatment in polycystic livers are carefully investigated. Likewise, the chance of adverse events and treatment efficacy need to be determined.[84]

In the research field of PLD, the introduction of the **PCK rat model** accelerated the study of renal and hepatic cystogenesis.[85, 86] This animal model harbors a spontaneous splice site mutation in the *Pkhd1* gene. This gene is orthologous to the human *PKHD1* gene causing ARPKD.[76] Polycystic kidneys and intrahepatic polycystic liver disease were characterized in the PCK rat.[87] This pre-clinical *in vivo* model showed a reduction of cyst volume upon somatostatin analogue octreotide administration.[88] The principle of octreotide treatment relies on binding on somatostatin analogue receptor 2 (SSTR2) which results in decreased cholangiocyte fluid secretion and inhibition of cAMP production.

This pre-clinical study resulted in further clinical research. Subsequently, a pharmaceutical treatment of **somatostatin analogues** was implemented in clinical trials. The first randomized placebo-controlled clinical trial including PCLD patients determined the total liver as primary outcome measurement. A significant polycystic liver volume reduction of 2.9% was reported by lanreotide administration during six months.[89]

In animal models only the effects on hepatic cystogenesis and liver volume of medical treatment is studied. A subset of PCLD patients reported no reduction of liver volume in clinical trials. In addition, discrepancies between the outcome of total liver volume and quality of life may occur. Several aspects may be related to treatment efficacy in PLD patients such as **environmental influences**, **genetic cause** and the involvement of multiple **biological pathways**. Therefore, the search for polycystic liver disease genes is required in order to elucidate target pathways for treatment.

Studies in 3D Cultures

An alternative approach to study PLD management are *in vitro* 3D cholangiocyte cultures of normal and PCK rats.[88] Recently, hyperactivity of matrix metalloproteinases (MMPs) in these *in vivo* and 3D cell culture models were assessed.[90] MMPs have critical roles in the embryological development, bile duct morphogenesis, tissue remodeling and breakdown of extracellular matrix. Altered MMP levels favor enhanced extracellular matrix degradation and triggers cyst formation.[91, 92] Therefore, a matrix metalloproteinase inhibitor, marimastat has been studied. The results show inhibition of cystogenesis in PLD models, but there are still questions about the dose administration, toleration and therapeutic efficacy in PLD patients. Although this research shed new light into the onset and evaluating the pathogenesis of PLDs in this research field, additional replication studies are recommended before approval into clinical trials.

The Relevance of PCLD Gene Identification

Disease gene identification may be beneficial for patients, their family members, clinicians and the society. Indications and ethical issues should be considered before early molecular testing in PCLD.

1) Detection of a pathogenic variant in a PCLD gene establishes a **definite diagnosis**. Few examples are;
   - Cases with usually severe or unusually mild disease manifestations,
   - Early-onset disease cases,
   - Solitary cases with a negative family history.
2) The identification of a disease-related mutation enables clinicians to **exclude ADPKD**, and progressive renal, multi-systemic disease.
   - This gives an accurate prognostic information to patients.
   - In ADPKD **screening** is advised for potential extra-renal disease to prevent vascular and cardiac events.[73, 93]
3) A genetic diagnosis provides (pre-)symptomatic PCLD family members with information regarding **family planning**.
   - Risk for transmission accounts 50% in autosomal dominant diseases.
4) A genetic diagnosis allows asymptomatic PCLD family members to obtain information about their **disease risk**.
   - Information and estimation about personal cost of mutation carriers.
   - Evaluation of potential living related kidney donors with doubtful imaging data, or individuals with a negative family history, and in cases of early-onset ADPKD.[37, 94]

In a **research** setting, there are other arguments to determine the causative gene. These scientific reasons aim for increased knowledge about molecular mechanisms, prediction of disease progression, assessment of genotype-phenotype relation and targets for treatment. Furthermore, mutation characterization of clinical trial cohorts may provide genetic stratification for the evaluation of such trials.[95]

Next-Generation Sequencing

Karyotyping, linkage analysis, homozygosity mapping and copy number variation (CNV) detection by genomic micro-arrays are established tools for disease gene identification. The search for genes associated with PCLD is mostly performed by conventional linkage analysis, usually based on rare large pedigrees.[30, 71, 72]

Since 2004, NGS is introduced and has revolutionized disease gene identification for Mendelian disorders.[96, 97] This type of massive parallel sequencing has become a more common instrument in genetic research.[98]

Targeted sequencing allows selective capture of a smaller number or single protein-coding genomic regions of interest. Polymerase chain reaction (PCR) is the most widely used enrichment. This approach is generally used for classical Sanger sequencing and is still highly effective to validate genetic variants, but can also be combined with NGS technologies for a limited number of amplicons.

Recently, several new techniques have been developed to more efficiently capture a larger number of exons or genes. One example for a medium scale of targeted genes (ca. 1-100 genes) is known as molecular inversion probes (MIPs).[99] This has been applied in terms of candidate gene analysis. Other techniques are either hybridization-based target enrichments or multiplexed PCR approaches. Probably the biggest advancement in disease gene identification is achieved by **whole-exome sequencing** (WES). This hybridization-based target enrichment technology allows sequencing of all protein-coding genes with only one reaction. The human exome consists of approximately 200,000 exons (1-1.5% of human genome). WES has led to a revolution in Mendelian disease gene identification.[98] Pathogenic variants in novel genes can be identified in single affected individuals with an extremely rare phenotype, but also in families with an unsolved monogenic disorder.[96, 101, 102]

Whole genome sequencing (WGS) provides the genetic information of the whole genome simultaneously in a single experiment.[102] Until now, this application has raised interest for disorders without identification of variants in the protein-coding part of the DNA. However, it can also provide the most uniform representation of the human exome as is does not show any enrichment biases. Another advantage is (almost) all types of genetic variants can be reliably detected in a single experiment including singe nucleotide variants (SNVs), small and large insertion-deletions (indels) as well as CNVs and other structural variants as inversions and translocations. The routine use of this technique is only a matter of time, until prices drop further and quality allows routine usage. Several population based genome studies are on their way, international cancer genome studies apply WGS and first clinical research applications have been recently shown.[103-105]

WES application in ARPKD

Although exome sequencing is the most powerful tool to study all genes at one time, it can also be effective for screening single or few large genes like the *PKHD1* gene in ARPKD.[106] The *PKHD1* gene has 67 exons, an open reading frame of ~12kb, and mutations may be distributed throughout the gene.[76] The majority of ARPKD patients have compound heterozygosity for *PKHD1* variants. Therefore, sequencing *PKHD1* in severe and mild phenotypes may efficiently confirm the diagnosis.

NGS applications in ADPKD

Because *PKD1* is a complex, large ADPKD gene, NGS technologies have already been applied in ADPKD cohorts.[66] The *PKD1* gene consists of 46 exons with an open reading frame of ~13kb and 50kb genomic DNA. In addition, both *PKD1* and *PKD2* present allelic heterogeneity. For *PKD1* raises another difficulty because of existence of six non-functional gene homologs (pseudogenes) that share 97.7% sequence identity with the *PKD1* gene (exon 1 to 33).[107] As a consequence pseudogenes often introduce errors in sequence datasets because of close homology to functional genes. It has been proposed that WES is applicable for screening unduplicated regions of ADPKD genes.[108] Currently, a long-range PCR (LR-PCR) enrichment followed by NGS has been reported for a high sensitive mutation-analysis in contrast to conventional Sanger sequencing.[109]

For example, a Chinese study has reported five novel *PKD1* variants detected by targeted NGS. *PKD1* and *PKD2* coding exons and flanking sequences were captured and barcoded before NGS in an Asian cohort.[110] These variants were validated in a high percentage of cases by direct Sanger sequencing. Here, NGS was ideal to detect mutations, and may represent an efficient tool in molecular diagnostics.

Another advantage of NGS based testing is the higher sensitivity for mosaicism, as was recently shown in a study of ADPKD patients with detection of *PKD1* mosaicism in an affected father. The 19-year-old daughter presented bilateral polycystic kidneys with a *de novo PKD1* mutation truncating polycystin-1. Long-range PCR and NGS for *PKD1* in the father's peripheral blood lymphocytes, buccal cells, sperm and urine sediment detected low levels (range 3-10%) of the mutated allele.[38]

WES or WGS in PCLD patients were not yet applied in research. Since PCLD is a monogenic disease expected to affect a protein expressed in the liver, application of WES is a rapid and straight-forward tool for disease gene identification.

Outline of this Thesis

For many years individual patient data regarding symptoms, radiological imaging and potential treatment are collected. Molecular studies are performed in our laboratory to obtain a definite diagnosis. Since the identification of the *PRKCSH* gene, and rapidly thereafter the *SEC63* gene, no other PCLD genes have been found.[30, 71, 72] Therefore, the genetic cause of hepatic cystogenesis remained unclear in the majority (~80%) of our PCLD patients.[28] For a better understanding and treatment of inherited diseases, a **comprehensive phenotype assessment** and identification of the cause is essential. The aim of this thesis is to identify the **genetic cause of disease** in PCLD patients in order to elucidate the pathogenesis of hepatic cysts. Therefore, I formulated different research questions.

I) *What are the current diagnostic criteria for adult polycystic liver disease?*
In **chapter 2** I present an overview of **adult polycystic liver disease phenotypes** including VMC, PCLD and ADPKD. Several classification systems and clinical manifestations are discussed in detail to verify the clinical spectrum. Secondly, I explored the current diagnostic criteria for PCLD and ADPKD in order to correctly assess radiological imaging and family history in our cohort. Patients with a **clinical diagnosis** of PCLD may be referred for molecular genetic testing of both known genes, *PRKCSH* and *SEC63*, for a definite diagnosis.

In contrast to ADPKD, there are no official guidelines for genetic counseling or screening family members in PCLD. In this case these aspects secondarily belonged to treatment and follow-up of patients and their families. In addition to complications and management of severely affected individuals, recommendations for (genetic) screening are outlined in this chapter.

II) *What is the phenotype of mutation-negative PCLD patients and their family members?*
Previously, the phenotype in PCLD patients with a *PRKCSH* (n=45) or *SEC63* (n=15) germline mutation (~20%) was assessed.[28] Investigations of a possible relation between genotype and phenotype revealed no significant results. The aim of this study was to assess the the clinical expression of the liver phenotype and inheritance pattern in mutation-negative PCLD patients. During this study we were able to screen family members for symptoms and phenotype by abdominal ultrasonography. In order to elucidate the **phenotype in mutation-negative PCLD families**, I performed clinical characterization of probands and family members (**chapter 3**). This assessment was indispensable during the search for novel PCLD genes as described in the next chapters.

III) *Are there more genes involved in PCLD?*
Although the primary aim of this thesis is the identification of novel genes associated with PCLD in this large cohort, mutation detection has only been performed by Sanger sequencing. This technique is recognized as the 'golden standard' to identify point mutations in genes, but is unable to detect genomic amplifications and deletions. In addition, the fact that there is no evident genotype-phenotype suggests allelic heterogeneity of PCLD.[28] Therefore, I hypothesized that a proportion of unlinked PCLD patients is explained by CNVs in the *PRKCSH* gene such as pathogenic deletions. I initiated this study to search for (large) duplications and deletions using MLPA analysis in **chapter 4**.

IV) *Can we use the mechanism of loss of heterozygosity to identify unknown PCLD genes?*
Hepatic cyst formation is ascribed to a cellular recessive mechanism. Our aim was to give a clear overview of the type and frequency in which somatic hits are reported in hepatic cyst tissue from ADPKD and PCLD patients. I performed a literature search in **chapter 5** and provide a comprehensive overview about this concept. In the following chapters 6 and 7, I applied different methods to identify polycystic liver disease genes.

Regarding the finding of a high frequency (78%) of somatic deletions in *PRKCSH*, I hypothesized that somatic loss of heterozygosity is a general mechanism in PCLD. The aim of this study was to identify genomic regions which may directly point to (candidate) polycystic liver disease genes. A protocol for **hepatic cyst epithelium cell-sorting** derived from collected

hepatic cyst fluid was designed. Subsequent DNA isolation, amplification and **genome-wide array based SNP genotyping** was performed to compare regions of homozygosity in cyst derived DNA with blood derived DNA. This allowed to study the presence of somatic cyst events in **chapter 6**.

V) *Is there another locus associated with PCLD?*
I describe an extended PCLD family without pathogenic variants in known PLD genes in **chapter 7**.

The clinical diagnosis of PCLD was obvious in about ~50% of family members and the pedigree clearly showed an autosomal dominant inheritance pattern in this family.

Whole-exome sequencing in two affected family members identified private variants (candidate genes) for PCLD in this family. Next, a candidate-based gene approach using the in-house bioinformatic pipeline was performed.[96, 97] Detected variants were subsequently prioritized, leading to the identification of ***LRP5* as a novel PCLD gene**.[111]

VI) *What is the role of LRP5 in ADPKD patients?*
As a logical consequence of the identification of *LRP5* associated with PCLD, I investigated the role of this gene in an ADPKD cohort. I hypothesized that *LRP5* may be involved as a causative or modifier gene. Therefore, we collected DNA from patients with a clinical diagnosis of ADPKD without pathogenic variants in *PKD1* and *PKD2*. **Direct Sanger sequencing** revealed *LRP5* variants in four ADPKD patients. These findings are outlined in **chapter 8**.

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PART I

POLYCYSTIC LIVER DISEASE PHENOTYPE

CHAPTER 2

POLYCYSTIC LIVER DISEASE: AN OVERVIEW OF PATHOGENESIS, CLINICAL MANIFESTATIONS AND MANAGEMENT

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ABSTRACT

Polycystic liver disease (PLD) is the result of embryonic ductal plate malformation of the intrahepatic biliary tree. The phenotype consists of numerous cysts spread throughout the liver parenchyma. Cystic bile duct malformations originating from the peripheral biliary tree are called Von Meyenburg complexes (VMC). In these patients embryonic remnants develop into small hepatic cysts and usually remain silent during life. Symptomatic PLD occurs mainly in the context of isolated polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD). In advanced stages, PCLD and ADPKD patients have massively enlarged livers which cause a spectrum of clinical features and complications. Major complaints include abdominal pain, abdominal distension and atypical symptoms because of voluminous cysts resulting in compression of adjacent tissue or failure of the affected organ. Renal failure due to polycystic kidneys and non-renal extra-hepatic features are common in ADPKD in contrast to VMC and PCLD. In general, liver function remains prolonged preserved in PLD. Ultrasonography is the first instrument to assess liver phenotype. Indeed, PCLD and ADPKD diagnostic criteria rely on detection of hepatorenal cystogenesis, and secondly a positive family history compatible with an autosomal dominant inheritance pattern. Ambiguous imaging or screening may be assisted by genetic counseling and molecular diagnostics. Screening mutations of the genes causing PCLD (*PRKCSH* and *SEC63*) or ADPKD (*PKD1* and *PKD2*) confirm the clinical diagnosis. Genetic studies showed that accumulation of somatic hits in cyst epithelium determine the rate-limiting step for cyst formation. Management of adult PLD is based on liver phenotype, severity of clinical features and quality of life. Conservative treatment is recommended for the majority of PLD patients. The primary aim is to halt cyst growth to allow abdominal decompression and ameliorate symptoms. Invasive procedures are required in a selective patient group with advanced PCLD, ADPKD or liver failure. Pharmacological therapy by somatostatin analogues lead to beneficial outcome of PLD in terms of symptom relief and liver volume reduction.

Keywords

Cystogenesis, Polycystic liver disease; Hepatomegaly; Biliary tract disease; Ductal plate malformation; Von Meyenburg Complex; Autosomal dominant polycystic liver disease; Autosomal dominant polycystic kidney disease

DISEASE NAME

Polycystic liver disease (PLD) is a collection of rare human disorders that result from structural changes in the biliary tree development.[1, 2] Genetic mechanisms and/or signaling defects are the root cause of ductal structures to become separated from the biliary tree finally resulting in cyst formation.[2, 3] Typically, these disconnected biliary structures are present in a very early disease stage, but remain asymptomatic until cyst growth initiates in adulthood.[4]

Three PLD entities are recognized in adults. Von Meyenburg complexes (VMC; biliary hamartoma; hepatic cystic hamartoma) with characteristic small, non-hereditary nodular cystic lesions [ORPHA386].[4, 5] Isolated polycystic liver disease (PCLD; autosomal dominant PLD) [OMIM#174050; ORPHA2924] with presence of innumerable hepatic cysts and autosomal dominant polycystic kidney disease (ADPKD) [OMIM#173900; OMIM#613095; OMIM#600666; ORPHA730] with cysts in both kidneys and in many cases hepatic cysts.

This paper reviews the pathological and clinical features of these three adult cystic disorders that share presence of numerous hepatic cysts with an intact biliary tree architecture.

DEFINITION

PLD is a rare inherited Mendelian disorder that is characterized by development of multiple hepatic cysts. The classification of PLD follows the histological changes that are due to ductal plate malformation (DPM) during fetal development.[6, 7] Definitions of cystic malformations are either based on the location of the affected (cilium-related) protein or follows radiological findings.[8, 9]

Ductal Plate Malformation

The biliary tree emerges from the endodermal hepatic diverticulum.[1] Development of the biliary system starts from the 8th week of gestation by formation of single layered hepatoblasts surrounding the portal vein (ductal plate). Duplication of ductal plate cells forms a double layer that finally dilate to a tubular structure, the primitive bile duct. Hepatoblast differentiation to a biliary phenotype and tubulogenesis is stimulated by the Notch, TGF-β and canonical Wnt signaling pathways.[2] Cell differentiation from hepatoblasts to cholangiocytes, tubule elongation and bile duct remodeling are completed by 30 weeks of gestation. Intrahepatic and extrahepatic bile duct systems are then merged and share the hepatic hilum. During the first year of life intrahepatic biliary epithelium maturates further.[1, 2, 7] PLD develops as a result of ductal plate malformation. The stage that is affected by faulty remodeling determines the phenotype. For example, VMC is thought to result from embryonic ductal involution at a late stage.[7, 10]

Bile duct formation requires a network of epithelial-mesenchymal interactions, and presence of growth and transcription factors to control appropriate cell migration, adhesion and cholangiocyte differentiation. Aberrant expression profiles and signaling result in deficient remodeling, and subsequently abnormal dilated or disconnected ductal plate cells developing into biliary cystic structures.[3, 11] Recently, a new classification for DPM has been proposed on the basis of *Hnf-1*β, *Hnf-6* and *cystin-1* gene deficient mouse models. This classification

distinguishes three DPMs: 1) abnormal hepatoblast differentiation, 2) failure of bile duct maturation, 3) perturbation of ductal expansion.[6]

Ciliopathy and Cholangiopathy

Ciliopathies represent an emerging class of human disorders that are caused by defects in distinct genes affecting ciliary structures or function. They may be inherited as simple recessive traits, but also in a dominant fashion. Phenotypic expressivity is under the control of numerous genetic modifiers.[8] Ciliopathies usually result in shared clinical features, such as intellectual disability, retinal defects and polydactyly, but the most well-known phenotype is that of cystic kidneys.[12] The proteins affected in ADPKD are located at the cilium which has led to the classification of ADPKD as a ciliopathy.[13] By contrast, the proteins associated with PCLD are not located to the cilium. Hepatic cysts are lined by cholangiocytes and therefore the term cholangiopathy is used for PCLD.[3]

Radiology

Radiological imaging assists in classifying PLD. Detection of macroscopically hepatic and renal cysts is facilitated by ultrasonography, magnetic resonance imaging (MRI) or computed tomography (CT)-scanning without or with (creatinine-permitting) intravenous contrast material.[9] On ultrasound, cysts appear as homogeneous anechoic fluid-filled round spaces. MRI is superior over ultrasound and CT, and allows better detection of small cysts in young individuals.[14] This technique captures biliary tree pathology and differentiates parenchym from biliary tree (Figure 1).

EPIDEMIOLOGY

PCLD has a prevalence of 1/100,000 to 1/1,000,000 or 1- 9/100,000 (1/158,000 in The Netherlands), while the prevalence of ADPKD ranges between 1/400 to 1/1,000.[13, 15] The incidence of VMC has been estimated up to 1/18-1/145 or 7-60/1,000 (0.69-5.6%) depending on the various autopsy studies.[10, 16]

CLINICAL DESCRIPTION

Von Meyenburg Complexes

VMC, also termed microhamartomas, are benign cystic nodules scattered throughout the liver. They are usually interlobularly located and at peripheral bile ducts below the Glisson's capsule.[7] VMC may occur isolated or in the context of PCLD and ADPKD.[4, 11, 17] VMC are frequently an incidental finding at radiological imaging, surgery or autopsy studies.

Histologically, they are characterized by small embryonic DPM remnants (<1.5-cm-diameter) or larger (small) hamartomas (>1.5 cm) delineated by regular cuboidal epithelium and embedded in fibrous stroma. Dilated structures initially communicate with the peripheral intrahepatic biliary tree, but separate with development.[7, 10]

VMC usually remain silent during life and require no management or follow-up examination. Although mild liver test disturbances may be observed, significant hepatomegaly or liver

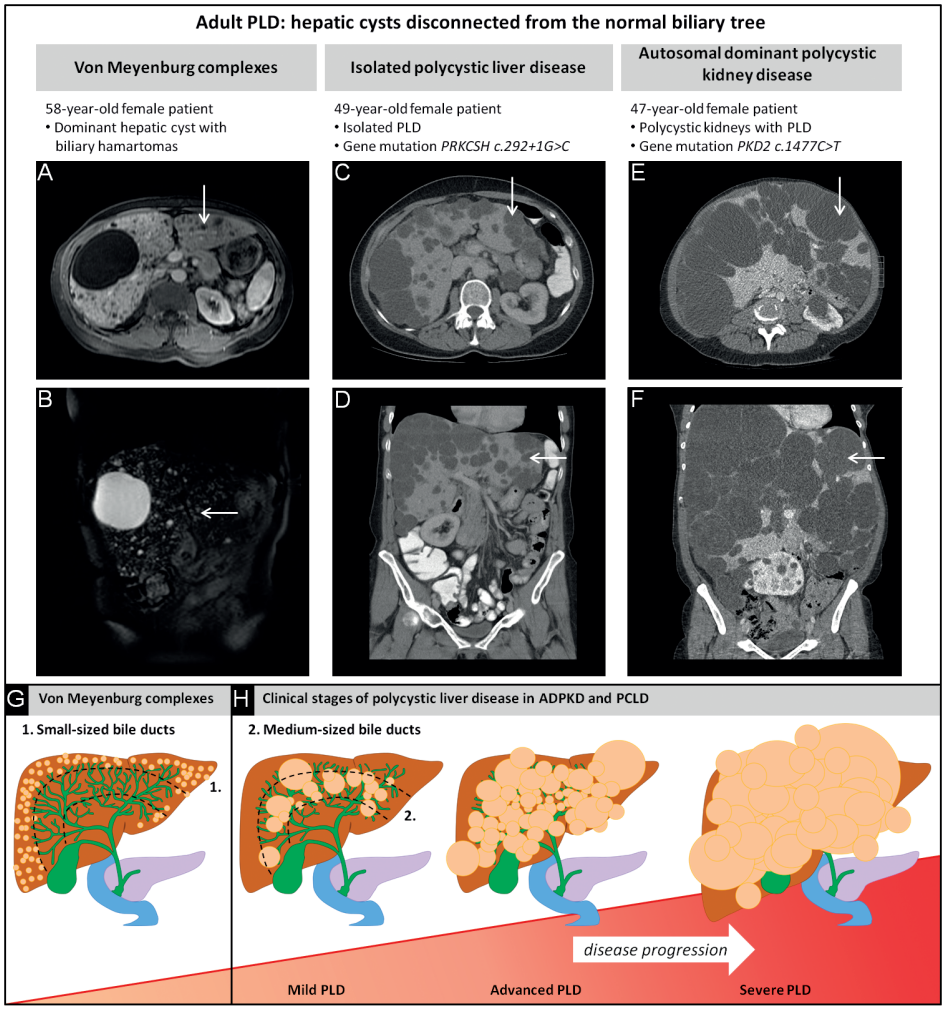

Figure 1. Abdominal MRI and CT in patients with PLD. (A) Axial T1-weighted and (B) coronal T2-weighted MRI present 1 large cyst and numerous cystic nodules scattered at peripheral bile ducts. (C-D) CT-scanning in a PCLD patient presents multiple cysts originating from medium-sized bile ducts. (E-F) Co-occurrence of polycystic kidneys exists in ADPKD. Both *PRKCSH* and *PKD2* gene mutations were predicted to be pathogenic (GRCh37-hg19; HGMD). Hepatic cysts are indicated by white arrows. (G) Diffuse VMC present numerous small-sized hepatic cysts located at peripheral branches of the biliary tree (in green). (H) The PLD phenotypes are arbitrarily staged and indicate disease progression. The disease course is progressive in a subset of severely affected PLD patients.

disease is rare in VMC. Incidentally, clinical features of epigastric pain, fever, cholangitis and jaundice appear when communication of multicystic VMC with the biliary tree cause biliary obstruction.[18] Episodes of liver sepsis indicates antibiotic treatment and follow-up of liver function tests. Abdominal pain and discomfort resolves with time. The diagnosis of VMC can be confirmed by MRI. Extra-hepatic features are absent.

PCLD and ADPKD

Hepatic cysts are the major clinical feature in PCLD and the most frequent extra-renal manifestation in ADPKD.[14] Cysts originate from medium-sized bile ducts. Histologically, they are delineated by cuboidal, flattened epithelial cells surrounded by fibrous stroma.[7]

They may be confined to one or more segments or spread evenly throughout the liver. Presence of large and numerous cysts frequently lead to hepatomegaly. One study showed that the overall hepatic cyst prevalence in ADPKD patients (age range15-46 years) is 83%, with the highest prevalence of 94% in 35- to 46-year-old patients. This corresponds with an increased prevalence of multiple hepatic cysts in older PLD patients.[14]

The variable number, size, location and distribution of cysts determines the spectrum of symptoms which is related to the extent of hepatomegaly.[4] Pain may ensue mainly because of tension on Glisson's liver capsule. Abdominal discomfort, pyrosis, early satiety, weight loss and anorexia may arise in advanced PLD.[15, 19] Patients who have large hepatic cysts that exert pressure on the stomach and displacement of other abdominal organs are at risk for malnutrition and nutritional deficiencies.[20]

Females with ADPKD or PCLD usually have a more severe liver phenotype, especially for those with a history of multiple pregnancies and prolonged exogenous estrogen exposure.[15, 21, 22] Females have higher average liver volumes, are younger at presentation and more susceptible to progressive PLD, suggesting a hormonal component.[14, 15, 19] In general, patients with PCLD have more severe symptoms and liver-related complications compared to ADPKD.[23] Hepatic cysts are rarely present in young children, but in exceptional cases symptomatic PLD may develop in young ADPKD patients.

The risk to develop severe PLD is independent from the ADPKD genotype, but is related to the severity of renal disease.[13, 23] Here, presence of a *PKD1* mutation puts the patient at risk for an earlier onset and outcome of renal disease compared to the *PKD2*.[24, 25]

Hepatic Complications in PCLD and ADKPD

Complications may occur due to massively hepatomegaly or result from invasive treatment.[15, 19] It has been estimated that about half of the patients with advanced hepatic disease have had cyst hemorrhage, cyst rupture or a cyst infection.[15, 26] These manifestations appear to be more frequent in ADPKD compared to PCLD.[27] Here, we will discuss the clinical and diagnostic signs for cyst hemorrhage, rupture, infection and other rare complications in PCLD and ADPKD.

Hemorrhage

Intrahepatic cyst bleeding typically presents as acute pain in the upper right-side of the abdomen. Symptoms develop acutely, progress in the first day(s), but resolve spontaneously. Sometimes, the nature of the abdominal pain can be colicky and accompanied by vomiting. Ultrasonography may be helpful to assess features suggestive for cyst bleeding such a higher attenuation value, aggregated fibrin deposits and possibly internal septa of hematomas. High signal intensity on MRI supports the diagnosis of an intrahepatic cyst bleeding.[9, 28]

Infection

Cyst infections are a serious complication because of its indolent course, demanding treatment and high risk of recurrence. Current diagnostic criteria rely on clinical, biological and radiological parameters including abdominal tenderness, fever (>38°C for >3 days), an increased C-reactive protein level and proven absence of spontaneous intra-cystic bleeding by CT-scanning. Hepatic cyst wall thickening and heterogeneous fluid (debris) are suggestive for infection. The diagnostic accuracy of MRI is unknown and CT-scanning has a low sensitivity and specificity to identify cyst infection.[29] ^{18}F-FDG positron emission tomography (PET)-imaging technique is preferable to detect the exact location of the infected lesion(s).[29]

PLD patients may have strongly elevated CA19-9 levels.[30] Extremely high CA19-9 levels were found in episodes with hepatic cyst infection and declining during recovery.[31] Detection of neutrophils and infectious agent(s) in the cyst fluid aspirate confirms the cyst infection and indicative for targeted antibiotic treatment.[29]

Rupture

Cyst rupture is an exceptional rare complication and presents with acute onset of pain. Hemodynamic complications are rare, but have been reported in the literature.[32] Abdominal discomfort from prolonged ascites and increased cyst volume needs stringent follow-up. If intra-peritoneal (blood) fluid leakage persists, a surgical intervention is inevitable for hemostasis control.[28]

Portal hypertension and ascites

In advanced stages there are two processes that may lead up to portal hypertension. First, there is reduction of hepatic vein outflow. Secondly, portal vein inflow may be compressed in advanced disease due to the volume effect of cysts.

Signs of hepatic vein outflow obstruction (HVOO) are abdominal pain, hepatomegaly and transudative ascites (90-96%).[33] Hepatic vein thrombosis is commonly recognized as the cause of HVOO, and case series reported also the Budd-Chiari syndrome secondary to PLD.[33, 34] The survival of hepatic vein thrombosis is low in severe cases.[35] Mechanical pressure symptoms of hepatomegaly may extend from hepatic veins to junctions with the vena cava inferior (IVC). Compression of IVC is characterized by increased renal outflow pressure that provokes development of ascites and edema in the lower extremities.[36]

In addition, hepatic cysts may cause a compromised portal venous flow.[37] These abovementioned complications lead to development liver fibrosis. Secondary complications of portal hypertension are the result of severe liver fibrosis such as esophageal varices, splenomegaly and transudative ascites may develop, but advanced fibrosis is a rare event.[26] Typically these features are seen in the elderly. In addition, lymphatic leak and chronic renal disease may contribute to development of ascites in severe PLD patients.[33, 37]

Jaundice

Portal hypertension may be accompanied by other signs of hepatic failure such as jaundice.[38] Although jaundice is usually seen in advanced stages, it may occur at any stage. An uncommon

cause of jaundice is obstruction of the intrahepatic or extrahepatic bile ducts by hepatic cysts.[26] Recurrent cholangitis is a rare complication of this condition.[34]

End-stage liver disease

Progression to end-stage liver disease usually results in the context of extremely increased liver volumes. Liver failure is seen incidentally, usually in a very late stage of the disease.[15, 19] Symptomatic patients with hepatomegaly (severe PLD) frequently do not meet the Model for End-Stage Liver Disease (MELD). Therefore, MELD exception criteria including assessment of malnutrition and quality of life are used.[20] Liver transplantation has excellent survival rates (>90% at 5 year).[39, 40]

Extra-Hepatic Manifestations

Kidney

The main differentiating feature between PCLD and ADPKD is the presence of polycystic kidneys. While this is the primary lesion in ADPKD, renal disease is absent in PCLD.[27] Disease progression depends on genotype, but also on environmental factors.[25] The majority of adult ADPKD patients develop enlarged kidneys and end-stage renal disease. In contrast, few renal cysts may be present in 28-35% of PCLD patients, but renal failure does not occur.[15, 19]

In contrast to PCLD, ADPKD is a multi-systemic disorder. Hypertension is one of the first signs of renal disease development and is related to progressive kidney enlargement and loss of renal function.[41, 42] It is still unclear whether early anti-hypertensive treatment prevents renal function decline.[43] The most common feature is abdominal/flank pain due to pressure symptoms and stretching of the renal cyst wall. ADPKD patients are also at-risk for other renal complications such as hematuria, urinary tract infections and kidney stones.[21, 41]

Cardiovascular system

ADPKD patients may develop hypertension, intracranial aneurysms (ICA), arterial aneurysms and several cardiac valvular abnormalities.[44] Early assessment of cardiovascular risk factors in ADPKD is advised, especially in young ADPKD patients.[45, 46]

Mitral valve prolapse has a higher prevalence in ADPKD up to 25-41.2% compared to 0-10.5% in PCLD.[19, 27, 47, 48] Other important connective tissue abnormalities such as aortic root dilatation and abdominal aorta aneurysm (AAA) have been reported in ADPKD.[49] If the family history is positive, screening of unaffected family members by MR-angiography is recommended.[50, 51]

Non-renal extra-hepatic cysts

The phenotype of PCLD is mainly restricted to the liver. Extensive radiological imaging of ADPKD patients have demonstrated that there are cysts in other abdominal organs such as the pancreas (9%) or seminal vesicles of the testis (43%).[52, 53] These cystic manifestations symptomatically silent.

Arachnoid cysts is central nervous system manifestation that is seen in 8% of ADPKD patients. This condition may occasionally lead to a subdural hematoma.[50]

Table 1. Genetic and diagnostic determinants of PCLD and ADPKD. A detailed overview of genotype characteristics and diagnostic criteria for PCLD and ADPKD phenotype assessment.

	Polycystic liver disease (PCLD)	Autosomal dominant polycystic kidney disease (ADPKD)	Reference
Genotype			
Cytogenetic gene location (mutation frequency %)	Chr.19p13.2: *PRKCSH* (15%) Chr.6q21: *SEC63* (5.7%)	Chr.16p13.3: *PKD1* (75.7%) Chr.4q21: *PKD2* (13.4%)	62, 78
Mutation (type; N, %)	25 ***PRKCSH*** 22 ***SEC63***	980 ***PKD1*** 193 ***PKD2***	[HGMD]
missense	4 (16%) 6 (27.3%)	250 (25%) 29 (15%)	
splice site	4 (16%) 3 (13.6%)	77 (7.8%) 32 (16.6%)	
insertion/deletion	10 (40%) 7 (31.8%)	440 (45%) 80 (41.5%)	
indel	1 (4%) -	13 (1.4%) 7 (3.6%)	
nonsense	6 (24%) 6 (27.3%)	202 (20%) 45 (23.3%)	
complex rearrangement	-	8 (0.8%) -	
Gene product; protein localization	**Cholangiopathy** Hepatocystin/ glucosidase II-β subunit; ER Translocation protein SEC63 homolog; ER, membrane complex	**Ciliopathy** Polycystin-1 (TRPP1); primary cilium, tight junction, extracellular matrix, ER Polycystin-2 (TRPP2); primary cilium, tight junction, extracellular matrix, ER	13, 60
Protein function	Proper protein folding and protein quality control Posttranslational protein transport	PC-1 and PC-2 form a mechanosensor complex on the primary cilium PC-1 for signaling detection, PC-2 is a TRP channel for calcium influx	60 13
Predominant phenotype			
Liver features			
PCLD diagnostic criteria: *Clinical practice:*	Positive family history with: <40 years and ≥1 hepatic cyst ≥40 years and ≥4 hepatic cysts 30-70 years and polycystic liver (>20 hepatic cysts)	Most common extra-renal manifestation: 83% with a polycystic liver (>20 hepatic cysts)	74 14
Kidney features			
ADPKD diagnostic criteria:	Incidental finding without renal disease: 28-35% with renal cystogenesis	Positive family history with: 15-39 years and 3 renal cysts[#] 40-59 years and 2 renal cysts* ≥60 years and 4 renal cysts* Negative family history with: <30 years and 5 renal cysts[$] 30-60 years and 5 renal cysts[$] >60 years and 8 renal cysts[$]	15, 19 76 75

Description of the data: A detailed overview of genotype characteristics and diagnostic criteria for PCLD and ADPKD phenotype assessment.
[#] unilateral or bilateral.
* in each kidney.
[$] bilateral.

Abdomen

Abdominal wall hernias may be present in PLD patients. Clinical series have suggested that (para)umbilical and inguinal hernias may be seen in up to 15-45% of ADPKD patients which may be explained by chronic compression due to high liver and kidney volumes.[27, 41, 54] Early reports have suggested a higher prevalence of colonic diverticuli in PLD, but upon scrutiny there was no evident association or increased risk for diverticular disease.[27, 55]

ETIOLOGY

Germline Mutation

Genetic analyses of both PCLD genes *PRKCSH* [OMIM*177060] and *SEC63* [OMIM*608648] may confirm the clinical diagnosis and differentiate it from other PLD (Table 2).[56-58] These genes encode for the (glyco)proteins hepatocystin and Sec63p.[59] Both proteins are located within the endoplasmic reticulum (ER) and are responsible for quality control and translocation of glycoproteins into the ER.[60] Since about 16-22% of PCLD patients harbor a pathogenic variant, PCLD is assumed to be genetically heterogeneous and other loci should be involved. There is no clear genotype-phenotype association.[15, 61]

Mutations in the *PKD1* gene [OMIM*601313] or *PKD2* gene [OMIM*173910] are responsible for renal cyst initiation in >90% of the cases.[62] Several ADPKD families possess no mutations on *PKD1* or *PKD2*.[63, 64] It is hypothesized that these cases are linked to another (yet unidentified) *PKD3* gene [OMIM*600666].[65] *PKD1* gene carriers have a higher prevalence of hypertension, complications and a higher risk of progressive renal failure compared to *PKD2*. Renal failure occurs at a much earlier age in *PKD1* carriers compared to *PKD2* carriers.[41]

Second-hit Hypothesis

PLD patients have a heterozygous germline mutation and it is hypothesized that cysts arise through functional loss of the second allele.[59] This is the rate-limiting step in the formation of cysts. Secondary, somatic hit mutations have been identified in *PKD1* or *PKD2* genes in liver and kidney tissues from ADPKD patients.[66, 67] Similarly, loss of heterozygosity of the *PRKCSH* or *SEC63* allele is present in PCLD cyst tissues. This is fitting with the second-hit-model for tumorgenesis which dictates that the combination of a germline and somatic mutations result in inactivated protein in target tissues.[68, 69]

Modifier Genes and Environmental Factors

Mouse models suggest that additional genes may lead to hepatic and renal cystogenesis.[6] It has been shown that *HNF-1*β mutations affect disease progression and outcome in ADPKD.[25, 70]

Once a cyst have been formed, progression to clinical significant disease requires other mechanisms.[8] Cyst fluid contains serum proteins but also cytokines and growth factors which contribute to cyst formation.[71, 72] Expression of estrogen hormone receptors in cyst epithelium may trigger growth advantages.[73]

DIAGNOSIS

VMC is a radiological diagnosis.[9, 10] Ultrasonography shows multiple hyperechoic areas in the subcapsular region. The comet-tail sign is a special form of reverberation artifact in detection of small cysts. This sign appears as a trail on the image if small calcific or highly reflective foci are interrogated on ultrasound.[5] MRI is preferred above CT-scanning as it readily shows multiple hyperintense focal lesions on T2-imaging.[9]

Current diagnostic criteria in PCLD and ADPKD rely on the age-related cystic liver phenotype with a positive family history of autosomal dominant inheritance (Figure 2).[74-76] Ultrasonography of the liver and kidneys is usually the first modality used to assess a cystic phenotype. The unified Ravine criteria for diagnosis of ADPKD relies on counting the number of renal cysts in at-risk individuals.[75, 76]

A single laboratory test that is of discriminative value for PLD is lacking. The synthetic liver function is usually preserved in PLD. Mild elevations of γ-glutamyltransferase sometimes combined with elevated alkaline phosphatase values may be detected.[15, 19]

DIFFERENTIAL DIAGNOSIS

PLD is a non-communicating biliary tree disorder and should be differentiated from other neoplastic, infectious and traumatic conditions.[4] The diffusion and variable small-sized hyperechoic structures in VMC are important to differentiate from hepatic metastasis or microabcesses.[9]

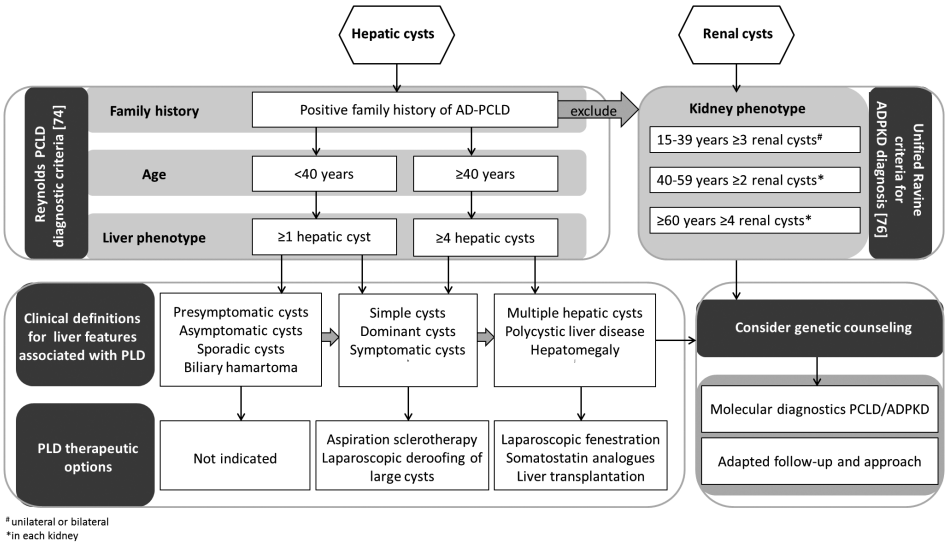

Figure 2. Comprehensive algorithm for diagnosis, management and genetic counseling in PCLD and ADPKD. The diagnostic criteria for PCLD and ADPKD compromises family history and age-related liver or kidney phenotype respectively.[74, 76] PLD therapy is individually decided according to number, distribution and size of hepatic cysts.[82] Genetic counseling has an important role in symptomatic individuals with a positive family history for hepatic and/or renal cystogenesis in order to differentiate PLD and clinical management. # Unilateral or bilateral; * In each kidney.

Table 2. Determinants and recommendations in severe PCLD and ADPKD. This scheme indicates distinct factors that are related to physical health and disease progression in PCLD and ADPKD patients. These clinical elements listed per organ may be valuable for consideration in management. Patients' mental condition and health-related quality of life should be assessed by standardized evaluation of symptoms in all PLD patients.[81]

Polycystic liver disease (PCLD)		
Organ	**Determinant**	**Recommendations**
Liver	Female sex Aging [21, 22] Environmental factors associated with PLD disease course: [21, 22] Prolonged oral/exogenous female steroid use: estrogens, contraceptive pill or (post-menopausal) hormonal replacement therapy Multiple pregnancies	Stop exogenous estrogen use in female patients [82] Advise alternative contraceptive strategies
Brain	Similar recommendations seem appropriate for patients with isolated ADPLD, but more studies are required [19, 77]	Indiscriminate screening is not recommended at present [44]
Heart	Similar as in the general population [47]	No recommendations
Autosomal dominant polycystic kidney disease (ADPKD)		
Organ	**Determinant**	**Recommendations**
Kidney	Environmental factors associated with renal cyst growth: [83] - caffeine - smoking Influencing factors for renal cystogenesis: [43, 45] - hypertension (≤35 years) - proteinuria - hematuria (<30 years) - urinary tract infection - kidney stones - renal infection - total kidney volume - male sex - low birth weight - aging *PKD1* gene mutation have a more severe disease course and earlier onset of end-stage renal disease compared to *PKD2* carriers [41]	Avoid (excessive) caffeine administration and nephrotoxic agents Smoking cessation Hypertension: [43, 45, 46, 80] - Routinely standardized blood pressure measurement - Elektrocardiogram in hypertensive patients for LVH assessment - Plasma LDL cholesterol control; urinary albumin excretion; left ventricular mass index calculation - Angiotensin converting enzyme inhibitors and/or angiotensin receptor blockers Dietary protein and salt restriction Sufficient daily fluid intake Molecular diagnostics [24]

In general, differentiation from communicating biliary tree disorders is important for management and prognosis. Bile duct ectasias or cystic dilatations belong to connected intrahepatic cystic diseases. These features are detected in ARPKD, Caroli disease (CD) and Caroli syndrome (CS). PLD is characterized by intrahepatic disease, a more late onset of disease in adulthood and absence of congenital hepatic fibrosis (CHF).

Table 2. ***Continued***

Organ	Determinant	Recommendations
Liver	Female sex Aging [21, 22] Environmental factors associated with PLD disease course: [21, 22] - prolonged oral/exogenous female steroid use: estrogens, contraceptive pill or (post-menopausal) hormonal replacement therapy - multiple pregnancies Renal function/glomular filtration rate [23]; in particular females [43]	Stop exogenous estrogen use in female patients [82] Advise alternative contraceptive strategies
Brain	Patients at risk: - positive family history of (ruptured) ICA or stroke <50 years old - previously ruptured ICA - warning symptoms: unusual headaches - high-risk occupation (for example: airline pilot) - preparation for major elective surgery (for example: kidney transplantation) [13, 51] The position of the mutation in *PKD1* is predictive for development of intracranial aneurysms [79]	Patients with reasonable estimated life expectancy: periodic 3-5 years MR/CT- angiography screening [51] Surveillance/rescreening after negative results in patients with a positive family history: 5-10 years (high- to low-risk) [13, 80] Smoking cessation Blood pressure control Hyperlipidemia control [51] Molecular diagnostics [79]
Heart	Screening is indicated: [13, 45] - a murmur or systolic clicks are detected on examination - positive family history of thoracic aorta dissection	Echocardiography [13, 45]
Aorta	ADPKD patients receiving hemodialysis [49] Similar as in general population for AAA: [80] - Male between the ages of 65-75 and smoked >100 cigarettes in a lifetime - Male >60 years and a family history of AAA	AAA: routine screening of the aortic size, using CT or abdominal ultrasonography [49] 1-time screening with abdominal ultrasonography [80]

Autosomal Recessive Polycystic Kidney Disease

Young and adult ADPKD patients are difficult to distinguish with other hepatorenal fibrocystic diseases such as ARPKD and other ciliopathies.[8] ARPKD has an incidence of 1/20,000 live births (ORPHA731; prevalence of 1.2/100,000) and a high peri-natal lethality. The predominant phenotype includes perinatal renal cysts and CHF.[12] Presence of cystic dilatation of intrahepatic biliary tree may be confused with disconnected hepatic cysts.[7] The gene product polyductin is located at the primary cilium suggesting molecular similarities with the ciliopathy ADPKD.[77]

Caroli Disease and Caroli Syndrome

CD is characterized by saccular, cystic dilations of the more larger intrahepatic biliary system. In CS large and small intrahepatic bile duct ecstasies are accompanied with CHF. CS has been typically associated with renal disease as in ARPKD.[7] The incidence of CD is approximately

1/1,000,000 births, but CS is more frequent (ORPHA53035; ~250 cases). Both ARPKD and CS have an autosomal recessive inheritance pattern.[12]

Genetic Counseling

Both PCLD and ADPKD have an autosomal dominant inheritance pattern and the recurrence risk is 50%. Genetic studies indicate an evident inter-familial clinical heterogeneity in PLD disease course among similar-aged patients. Secondly, intra-familial studies suggested a considerable phenotypic variability of hepatic cysts (Figure 3). Clinical asymptomatic or undiagnosed members contribute to underestimation of the actual disease prevalence.[24, 41, 61] It is estimated that the penetrance is ~80%.[78]

These considerations raise the question whether it is appropriate to screen members or children at-risk. Counseling should include discussion about insurance, employment and psychological factors. Genetic counseling is recommended in severely affected PLD and may afford differentiation between ADPKD and PCLD.[8, 12]

Molecular diagnostics may assist the counseling process to establish a firm diagnosis in symptomatic patients and families. In particular ADPKD, determination of the responsible gene is useful for those who are at-risk in order to develop a strategy to prevent severe progressive disease events or complications.[79] If the family history for ICA is positive in an ADPKD family, screening of unaffected family members by MR-angiography is recommended.[50, 51, 80] Counseling or genetic testing is not advised in asymptomatic children.[77]

Management and Prognosis

VMC is an asymptomatic condition without long-term consequences and treatment is not warranted. The primary aim of PLD therapy is to reduce symptoms by curtailing hepatic cyst development. The treatment of choice is driven by individual complaints.[40] Although the primary outcome measurement of PLD management is liver volume, assessment of symptoms associated with quality of life is an element for focus.[81] Therapeutic interventions are not warranted in asymptomatic patients.

The first advice in PCLD and ADPKD is to stop oral contraceptives.[20, 82] Although not formally investigated, the use other (non-systemic) contraceptives such as an intra-uterine device may be an acceptable alternative. Other guidelines are presented in Table 1.[80, 83] Supportive management with analgesics is the first-line treatment in patients with acute or chronic abdominal pain and tenderness.

The different invasive approaches with possible beneficial outcomes in independent studies include aspiration sclerotherapy, laparoscopic cyst deroofing or liver transplantation.[39, 40, 84] Current indications and considerations for invasive treatment are presented in Figure 4.

Treatment of portal hypertension and ascites are not different from that in patients with other causes.

In a selected patient group were invasive procedures such as a vascular or bile duct stent placement optional for decompression of the portal vein, inferior vena cava or bile duct, or in HVOO treatment.[37, 85] In general, management of vascular and bile duct complications due to hepatic cystogenesis consists of relieving the obstruction in order to improve venous and

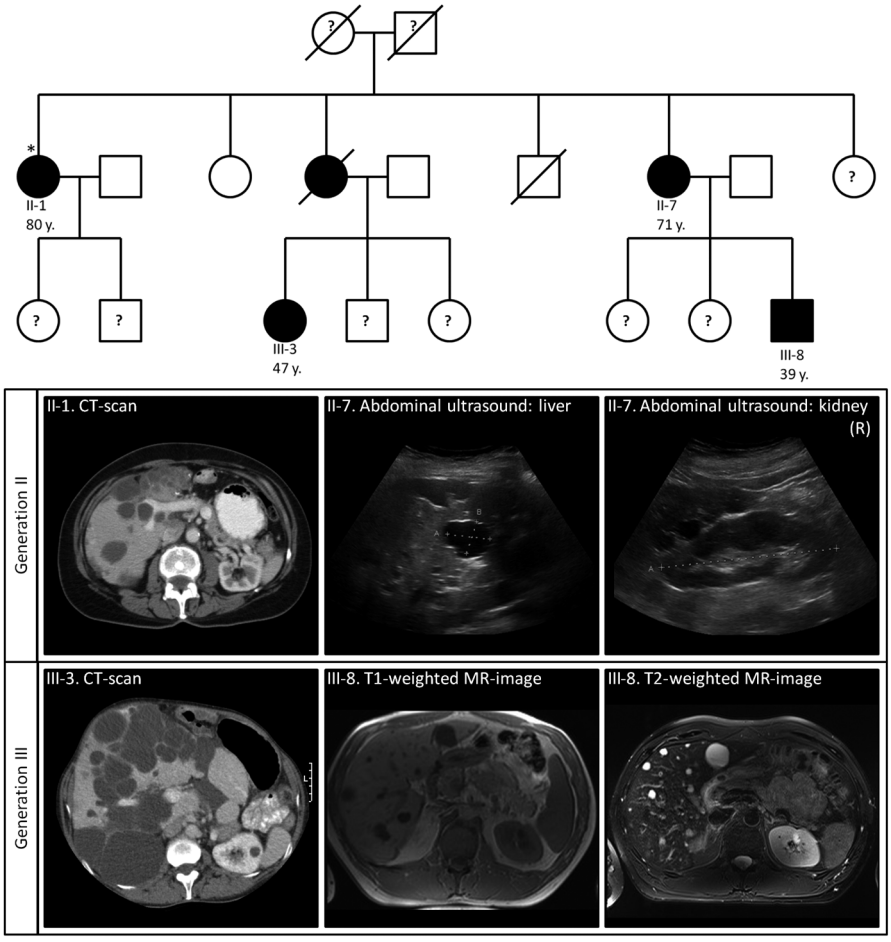

Figure 3. Intra-familial clinical heterogeneity in PCLD. Description of the data: (A) Pedigree of a PCLD family with *PRKCSH* gene mutation *c.374_375delAG* in affected individuals. The index patient (*) has 4 members with symptomatic PLD. Although the family history is positive, family members frequently are asymptomatic carriers or the liver phenotype remains unknown. (B) Axial CT-scanning, abdominal ultrasonography or MRI in four PCLD patients presented a variably number of hepatic cysts without renal disease.

biliary drainage.[26, 35] Although no concise guidelines are available, stents may give temporal symptom relief of portal hypertension, ascites and jaundice. A porto-systemic shunt may be indicated in presence of acute thrombosis or vascular compression to establish patent hepatic and portal venous flow, but should be weighed against possible complications. The primary aim is to ameliorate symptoms by cyst decompression in these advanced cases. This is often achieved by final treatment strategies including liver resection, hepatic fenestration procedures or liver transplantation.[33]

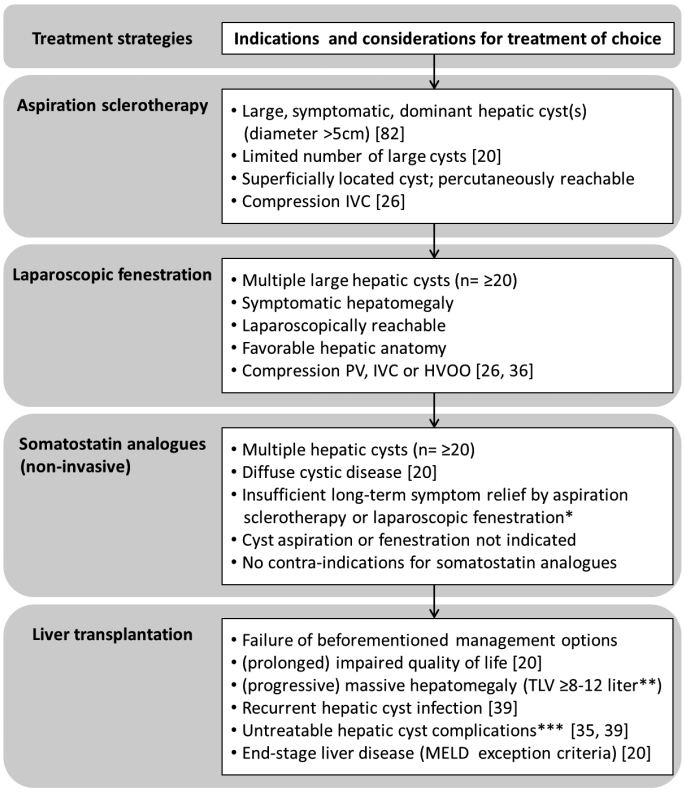

Figure 4. Indications and considerations for treatment strategies in symptomatic PLD. Schematic overview of relevant indications and considerations in PLD management. Patient characteristics and liver phenotype such as severity of clinical symptoms, age, (surgical) history, degree of hepatomegaly, and number, size and location of hepatic cysts are essential aspects for this decision. In general, the choice of procedure is an individual process in consultation with the patient. Severely affected individuals are patients with massive hepatomegaly, refractory symptoms, PLD-related complications and/or end-stage liver failure. Care and follow-up of these patients should be managed with caution. *Notification: Results about treatment efficacy of prolonged or long-term somatostatin analogue use is unknown. **Arbitrary total liver volume (TLV), because this outcome measurement is related to other parameters such as height, total body surface and sex. ***For example: recurrent hepatic cyst infection, HVOO.

Recent development of pharmacological options opened up new treatment strategies for severe PLD patients. Long-term follow-up studies with somatostatin analogues demonstrated that these agents consistently lower total liver volume in PLD patients.[86, 87] A recent meta-analysis reported that somatostatin analogues is particularly effective in young females.[88]

CONCLUSION

PLD compromises a clinically heterogeneous liver phenotype identified in VMC, ADPKD and PCLD patients. Massively enlarged livers are present in a subset of ADPKD and PCLD. Genetics and environmental factors such as exogenous estrogen intake and number of pregnancies

contribute to disease progression. A considerable intra-familial variability in liver phenotype and extra-hepatic features makes screening modalities uncertain in PCLD. Evaluation of PLD-related symptoms and quality of life are necessary to decide beneficial management.

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CHAPTER 3

HIGH CLINICAL HETEROGENEITY IN MUTATION-NEGATIVE ISOLATED POLYCYSTIC LIVER DISEASE FAMILIES

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Submitted

ABSTRACT

Isolated polycystic liver disease (PCLD) is characterized by a spectrum of symptoms due to hepatic cystogenesis ensuing hepatomegaly. Phenotypic expression and clinical features of cysts among relatives remain unclear.

PCLD patients were screened for *PRKCSH* and *SEC63* gene mutations. Family history and symptoms were assessed in probands at diagnosis. Liver and kidney phenotype among relatives were determined by abdominal ultrasonography combined with archival data.

467 unrelated patients were referred for PCLD mutation analysis between 2003 and 2014. In 21.6% the diagnosis was molecularly confirmed (15.2% *PRKCSH*; 6.4% *SEC63*). Consent was given by 162 relatives of 32 mutation-negative PCLD patients. A negative family history was reported in 24 families (75%), but cysts were detected in members of 22 families (91.7%).

The majority of PCLD patients without a pathogenic variant in the *PRKCSH* or *SEC63* gene present a false negative family history. Intrafamilial variability of the liver phenotype argues for multiple genetic and environmental factors associated with hepatic cystogenesis.

Keywords

Polycystic liver; Phenotype; Symptoms; Family history

INTRODUCTION

Polycystic liver disease (PLD) is a heterogeneous disorder that carries a significant morbidity and puts a social burden on patients, family members and environment.[1] PLD is part of the group of fibrocystic diseases which has two major representatives: isolated polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD).[2] Intrahepatic bile duct dilatation, hepatic fibrosis (Caroli syndrome) in combination with hepatic cysts may be present in autosomal recessive polycystic kidney disease (ARPKD).[3] These three Mendelian disorders share the presence of hepatic cystogenesis, but ADPKD and ARPKD patients also have polycystic kidneys. As a consequence, renal disease is the dominant feature in these patients.[3, 4] In addition, they differ in genetic background. About ~20% of unrelated PCLD patients harbor a mutation in the *PRKCSH* or *SEC63* gene.[5] Recently, four unique *LRP5* variants were identified in four unlinked PCLD families.[6] This contrasts with the situation in ADPKD where *PKD1* or *PKD2* germline mutations are found in most ADPKD probands (89%).[7] Likewise, most ARPKD patients have two *PKHD1* germline mutations (76-87%).[8, 9]

The position of germline mutations and the effect on protein function correlate with age of onset of end-stage renal disease in ADPKD.[10] On the contrary, clinical studies have established considerable variability of the renal phenotype in large *PKD1* [10] and *PKD2* families.[11] Affected ADPKD relatives differ in age of disease onset, symptoms and renal function.[10, 11] In ARPKD there are notable differences with respect to the presence of liver fibrosis and biliary disease among families.[3, 12] Here, the wide intrafamilial spectrum of phenotypical manifestations is ascribed to the large size of *PKHD1* with a large number of mutations and multiple alternative transcripts.[12]

Reports about the presence of intrafamilial heterogeneity in PCLD patients are not available. It has been suggested that hepatic cysts are undetected or not carefully assessed by radiological imaging.[5] In line with ADPKD and ARPKD family studies, we hypothesized that the clinical expression of the liver phenotype is not uniform among individuals from a given PCLD family.

METHODS

Study Protocol

We performed a cross-sectional study in our PCLD cohort by a comprehensive genetic and clinical analysis. For this family study we included probands with a clinical diagnosis of PCLD, which was unconfirmed by genetic analyses of *PRKCSH* and *SEC63* genes. All probands possessed a polycystic liver without renal disease and did not meet the Ravine criteria.[13] Eligible mutation-negative families consisted of at least two individuals in order to assess the familial PCLD expression profile. A detailed family history was assessed in those probands without a *PRKCSH* or *SEC63* gene mutation. Demographic characteristics are presented as median (range) and percentages.

PRKCSH and SEC63 Mutation Analysis

Blood samples (10 ml EDTA-anticoagulated) were collected from probands. DNA was extracted from blood leukocytes using the HP-PCR Template Preparation kit (Roche Applied Science,

Mannhein, Germany). Subsequently, molecular analyses of the *PRKCSH* and *SEC63* gene were conducted by direct Sanger sequencing on ABI3730 Genetic Analyzers (Applied Biosystems).

PCLD Relatives

All available first- and second-degree adult relatives were invited to participate. Enrolled were individuals willing to provide clinical data after written informed consent (Figure S1). PCLD is defined as presence of at least 4 hepatic cysts in individuals >40-years-of-age and a positive family history with a autosomal dominant inheritance pattern.[14]

Abdominal Ultrasound Examination

We determined the hepatic and renal phenotype in family members ≥35 years-of-age by abdominal ultrasonography combined with archival radiological data. Ultrasound images of liver and kidneys were acquired using a 3.6MHz general-purpose clinical echo system (Acuson x150, Siemens AG, Germany) equipped with a curved linear array transducer. Presence or absence of hepatic and/or renal cysts was carefully assessed by at least two clinicians (W.R.C/M.C./J.P.H.D.). Phenotype screening was performed in all relatives within 8 months.

RESULTS

The study population consisted of 467 unrelated new patients with PLD referred for molecular diagnosis of PCLD in the time span 2004-2013. In 21.6% of the patients Sanger Sequencing of *PRKCSH* and *SEC63* revealed the underlying pathogenic mutation (15.2% *PRKCSH*; 6.4% *SEC63*).

For this study, we included 32 accessible and unlinked probands for clinical assessment. Subsequently, a detailed family history was inquired. This group consisted of 29 female probands (90.6%) and had a median age of 51 years (range 26-75) at clinical diagnosis. In total, 162 family members consented for assessment of medical history and phenotype screening.

We systematically questioned relatives for symptoms prior to phenotype screening. These relatives were triaged according to a positive or negative family history and distributed among two groups. A positive family history of hepatic cystogenesis at diagnosis was confirmed by assessment of 59 relatives (37 affected and 22 non-affected) from 8 probands (25%). Twenty-four probands reported a negative family history at diagnosis (75%), but after screening there were hepatic cysts (range 1-≥20) present in family members from 22 probands (91.7%). A cystic phenotype was absent in family members of two probands with a negative family history.

After clinical assessment of our cohort probands with a negative family history consisted of 49 individuals with cysts and 54 unaffected family members. Affected individuals had a median age of 60 years (range 37-83) compared to 53 years (range 35-82) of non-affected individuals. Affected family members from probands with a negative family history of PCLD were mainly female (79.6%; median age of 57 years; range 38-83).

Five out of 22 families with an apparent negative family history had 1 affected family member with PCLD-related symptom(s) (Figure 1). They were all first-degree relatives (2 mothers, 2 brothers, 1 sister) with median age of 69 years (range 44-74). None of these five

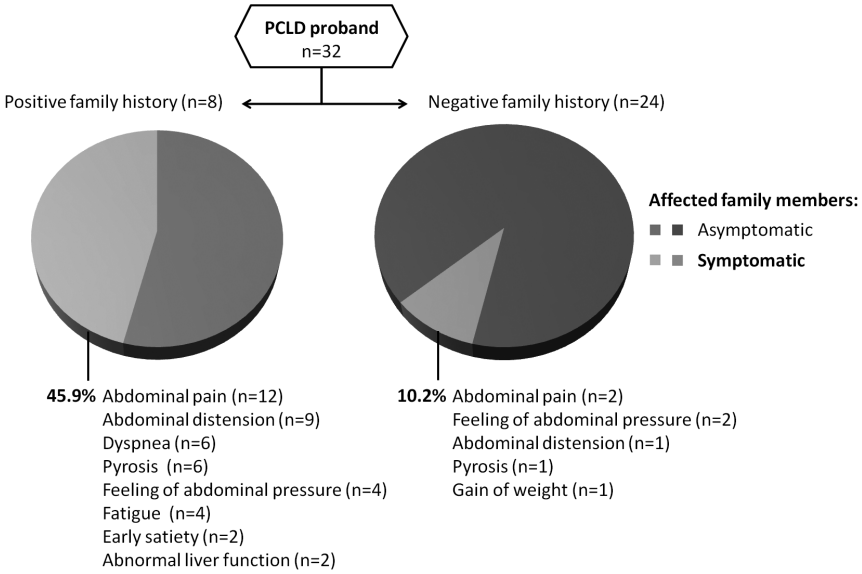

Figure 1. Results of the family study. Mutation-negative PCLD probands with a positive family history have almost equal asymptomatic (54.1%) and symptomatic (45.9%) affected family members. Five affected relatives of a proband with a negative family history presented PCLD-related symptoms (10.2%).

relatives were treated as having PCLD, while 83.3% of the probands was treated by one or more of the following therapies: aspiration sclerotherapy (n=17), laparoscopic deroofing (n=4) and/or somatostatin analogues (n=5).

DISCUSSION

This cross-sectional study establishes that solitary PCLD cases without a *PRKCSH* or *SEC63* germline mutation and no apparent family history frequently do have family members with bonafide PCLD. The phenotypes of index patients were in great contrast to those of new discovered affected family members. Astute clinical ascertainment established that the index patients were the most severely affected individuals within the family. Indeed, most index patients (83.3%) had sought medical care due to symptoms. We also found that there was great variability of the polycystic liver phenotype (in terms of number and size of cysts) detected in affected family members. Only few affected, disease-unaware family members (10.2%) had symptoms fitting with PCLD at the time of screening. Therefore, taking family history accurately in patients with unexplained abdominal symptoms may provide a broader diagnostic frame.

There are no established clinical criteria for diagnosis of PCLD. Hitherto, most researchers use the Reynolds criteria that comprises age, quantitative radiological findings combined with a positive family history following an autosomal dominant inheritance pattern.[14] Specifically, individuals who are above 40-years-of-age with at least 4 hepatic cysts and stem from a

PCLD family can be labeled with PCLD.[14] Symptoms are not part of this diagnostic set of criteria. However, symptoms such as abdominal pain and/or distension, and early satiety are not infrequent [1] and are part of the diagnostic process. Severe PCLD entails a syndrome of hepatomegaly and abdominal symptoms. Following this definition, a minority of inherited PCLD patients who meet the Reynold's criteria have symptoms. Our study presents that screening individuals within a family with PCLD on the basis of symptoms will detect ~50% of patients.

How do patient's symptoms relate to the ADPKD phenotype, whose patients have polycystic kidneys and may have polycystic livers? In ADPKD, patients progress to renal impairment. The severity of polycystic liver disease is correlated with renal disease progression in females according to studies measuring total liver and kidney volumes.[15, 16] There is an ADPKD genotype-phenotype correlation, as patients with *PKD1* gene mutations reach end-stage renal failure ~20 years earlier than *PKD2* mutation carriers.[17] Evidence exists for significant disease variability within ADPKD families that carry the same mutation.[11] This substantial disease variability includes non-progressive and progressive cases within one generation. The reason for such inconsistent presentation remains unexplained, but may partially result from genetic factors such as mutation type or presence of chromosomal aberrations (e.g. mosaicism).[10, 11] No genetic factors predict development of (severe) polycystic livers in ADPKD patients. In addition, there may be involvement of multiple (modifier) genes or environmental factors that contribute to intra- and interfamilial variation.

The unified ultrasonographic criteria for ADPKD diagnosis excludes the medical family history as diagnostic criteria in cases with an unknown genotype.[13] In addition, sporadic ADPKD patients may also harbor a *PKD1* or *PKD2* mutation, the only definitive diagnosis.[18] Current guidelines advise to consider screening members for ADPKD after identification of the index patient, even in absence of an apparent family history. This is justified in the context of the risk for extra-renal (vascular) complications.[19] We feel that in view of the absence of clear extra-hepatic manifestations, genetic counseling is not warranted in PCLD. However, we would like to emphasize that early diagnosis in young females may offer advantages, as these patients should be advised to avoid exogenous estrogens as these have a growth inducing effect on the polycystic liver.[2, 20] Radiological screening of young individuals (18-years-old or younger) is not indicated. In case of symptomatic PCLD patients, an extended first assessment is recommended: physical examination, taking family history, molecular analysis of the *PRKCSH* and *SEC63* gene and abdominal ultrasonography. CT scanning may be considered to have a radiological presentation as a reference (first measure point). Clinical follow-up and ultrasonography once a year may be advised in symptomatic patients, but patients with progressive disease need more frequently clinical monitoring. We believe that asymptomatic individuals require no extended follow-up program.

The strength of this study is the comprehensive analysis of the families of *PRKCSH* and *SEC63* negative index patients. We performed ultrasonography of the liver and both kidneys, and analyzed additional archival radiological data. Although abdominal ultrasonography among at-risk members is the first investigation of choice in a screening setting [13, 21], this study is limited by absence of total liver volume measurement.

In conclusion, this study shows the presence of significant intrafamilial, phenotypic heterogeneity in PCLD patients without a pathogenic variant in the *PRKCSH* or *SEC63* gene.

An extended family history may provide arguments for abdominal disease. We advise to perform abdominal ultrasonography as first screening tool in symptomatic individuals. Although (hormonal) environmental influences are part of the disease, the presented phenotypic variability also suggests heterogeneity at a genetic level.

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SUPPLEMENTARY FIGURE

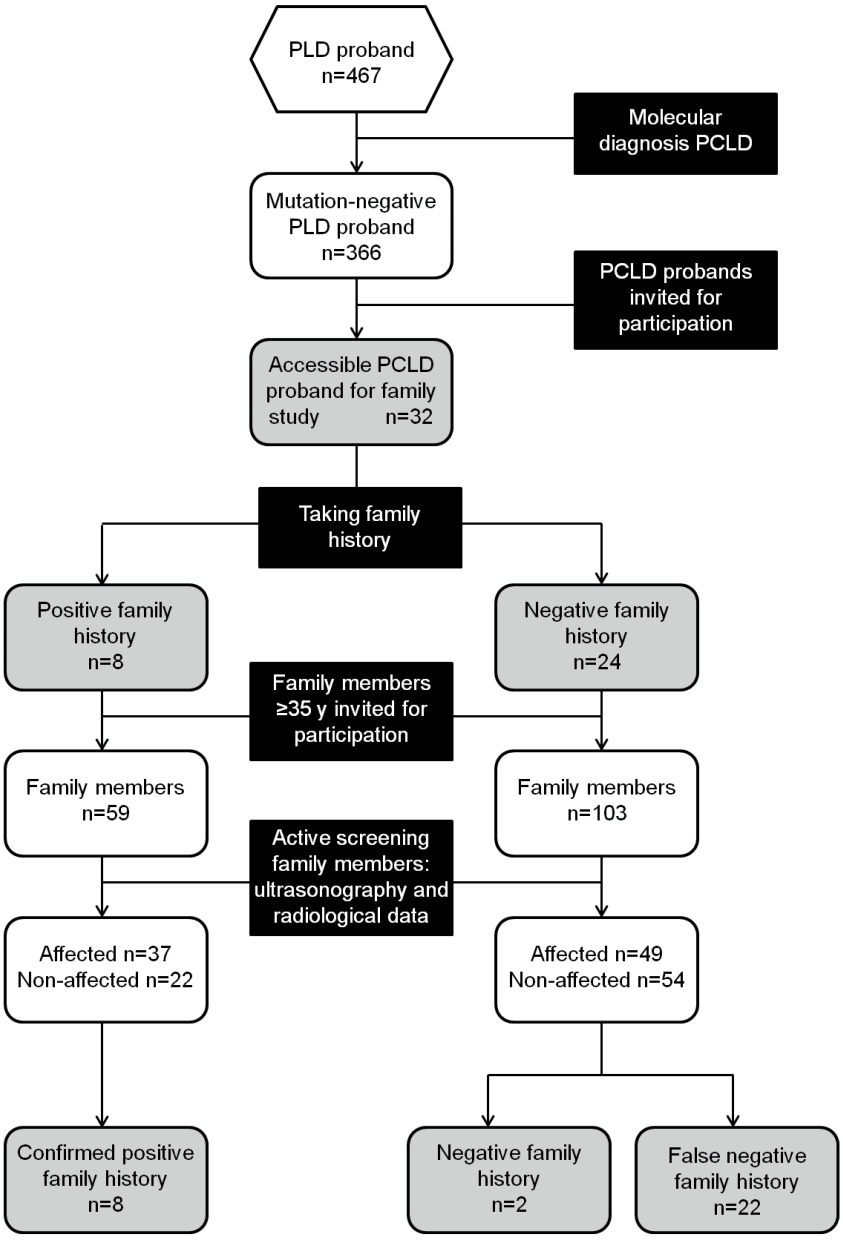

Figure S1. Flowchart recruiting and including families. The flow chart of this study presents that 21.6% of probands harbor a mutation in the *PRKCSH* or *SEC63* gene. Recruitment of probands and family members are schematically depicted.

PART II

GENETIC RESEARCH FOR POLYCYSTIC LIVER DISEASE GENES

CHAPTER 4

SEVERE POLYCYSTIC LIVER DISEASE IS NOT CAUSED BY LARGE DELETIONS OF THE *PRKCSH* GENE

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ABSTRACT

Isolated polycystic liver disease (ADPLD) is an autosomal dominant Mendelian disorder. Heterozygous *PRKCSH* mutations are the most frequent cause. Routine molecular testing using Sanger sequencing identifies pathogenic variants in the *PRKCSH* (15%) and *SEC63* (6%) genes, but about ~80% of patients meeting the clinical ADPLD criteria carry no *PRKCSH* or *SEC63* mutation. Cyst tissue often show somatic deletions with loss of heterozygosity which was recently recognized as a general mechanism in ADPLD. We hypothesized that germline deletions in the *PRKCSH* gene may be responsible for hepatic cystogenesis in a significant number of mutation-negative ADPLD patients.

In this study, we designed a multiplex ligation-dependent probe amplification (MLPA) assay to screen for deletions of *PRKCSH* exons. Genomic DNA from 60 patients with an ADPLD phenotype was included. MLPA analysis detected no exon deletions in mutation-negative ADPLD patients.

Large copy number variations on germline level are not present in patients with a clinical diagnosis of ADPLD. MLPA analysis of the *PRKCSH* gene should not be considered as a diagnostic method to explain hepatic cystogenesis.

Keywords

Exon deletion; Genetic heterogeneity; Hepatic cystogenesis; MLPA; ADPLD; *PRKCSH*

INTRODUCTION

Polycystic liver disease may occur in the context of isolated polycystic liver disease (ADPLD) and as a common extrarenal manifestation in autosomal dominant polycystic kidney disease (ADPKD).[1, 2] Polycystic livers have numerous cysts which may result in hepatomegaly.[1] While ADPLD is rare, 1/158,000, ADPKD is seen in 1/400-1/1,000.[3, 4] In addition, autosomal recessive polycystic kidney disease (ARPKD) patients are also predisposed to hepatobiliary abnormalities.[5]

A heterozygous mutation in *PRKCSH* (MIM*177060), *SEC63* (MIM*608648) or *LRP5* (MIM*603506) underlie ADPLD.[6-9] Currently, 27 *PRKCSH*, 22 *SEC63* and 4 *LRP5* mutations have been identified by conventional Sanger sequencing, with the highest mutation frequency of the *PRKCSH* gene (15%).[10-12] The mutation spectrum consists of heterozygous duplications, small deletions, missense, nonsense, and splice site mutations.[10, 12] Interestingly, 79-84% of patients with a clinical diagnosis of ADPLD do not harbor a pathogenic mutation on these loci.[10, 12] The overall low mutation detection rate in ADPLD may be explained by genomic rearrangements missed by clinical testing. Recently, somatic mutation analysis reported loss of the wild-type *PRKCSH* allele in 76% of micro-dissected cysts.[13] Extended loss of heterozygosity (LOH) was confirmed by investigation of the genomic region surrounding *PRKCSH*. Analysis for large deletions, mosaicism or epigenetic mechanisms such as promoter hypermethylation in germline DNA have not been performed for ADPLD.

We hypothesized that aberrations in genomic DNA in and close to the *PRKCSH* gene may be responsible for hepatic cystogenesis in mutation-negative patients with an ADPLD phenotype. In this study, we designed probes for the *PRKCSH* gene and performed multiplex ligation-dependent probe amplification (MLPA) assays in order to identify chromosomal deletions or duplications.

MATERIALS AND METHODS

Patient Samples

We selected individuals with severe polycystic liver disease who were referred to us between 2004 and 2012 for molecular analysis of the *PRKCSH* and *SEC63* gene.[12] In total, 60 DNA samples belonging to 56 families were analyzed by MLPA. This included six family members from two families with a known pathogenic *PRKCSH* variant causing ADPLD, which was absent in two affected family members.

Thirty-nine patients were affected with >20 hepatic cysts and 21 patients presented <20 large, dominant hepatic cysts. The mean age was 54 years (range 32-79) and 41 patients required treatment because of symptomatic ADPLD. Invasive treatment such as aspiration sclerotherapy and/or surgery was performed in 36 patients (60%). Somatostatin analogues was given to 13 patients (21.7%; mean liver volume 5,402 liter) during clinical trials.[14-17]

Isolation Genomic DNA

Genomic DNA was isolated from peripheral blood using the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). To yield a high DNA concentration, RNase was used to remove all RNA in the blood samples, and DNA was eluted with 100µl elution buffer.

Direct Sanger Sequencing

Conventional PCR mutation detection was conducted with specific primers for *PRKCSH* and *SEC63*. All 18 *PRKCSH* exons, 21 *SEC63* exons and flanking intronic sequences were screened by bidirectional Sanger sequencing on ABI3730 Genetic Analyzers (Applied Biosystems, Waltham, Massachusetts, USA).[12] Amplified fragments were purified using the QIAEX-II Gel Extraction Kit (Qiagen, Hilden, Germany).

PRKCSH MLPA Probe Set Design

The longest open reading frame of *PRKCSH* consists of 1,584bp with the start codon located in exon 2 and stop codon in exon 17 (DNA Genebank accession number: NM_002743.3; and genomic DNA Genebank accession number: NC_000019.9.[18] Therefore, genomic sequence of 16 coding exons of the *PRKCSH* gene and the first exon of the nearby gene in opposite transcriptional direction, exon 7 of the *ELAVL3* gene, were retrieved for probe design (Figure 1A). In total, the design strategy consisted of 17 synthetic left probe oligonucleotide (LPO) and right probe oligonucleotide (RPO) without stuffer sequences according to manufacturers' instructions; criteria by MELTingeny software v1.0.1 (Ingeny, Goes, The Netherlands) (Table S1).

We excluded two exons (11 and 16) of the *PRKCSH* gene because of conservation rates and extent of the kit (MRC Holland, Amsterdam, The Netherlands). In addition, both exons are flanked by the shortest intronic regions. Exon 11 is located 277bp and 96bp from exon 10 and exon 12 respectively. The intronic regions adjacent to exon 16 are even smaller, consisting of only 87bp and 88bp. Exon 11 is not highly conserved, contains several polymorphisms and no pathogenic mutations were identified in this region. Solitary splice site variants were identified in exon 16.[10] These facts implicate that these exons are probably not the hotspots for mutations involved in ADPLD development. Moreover, the probe design options for exons 11 and 16 were not in line with the high quality design compared the other developed *PRKCSH* candidate probe set.

To reduce presence of SNPs and for melting temperature analysis, candidate sequences were analyzed by SNPcheck v.3 (https://ngrl.manchester.ac.uk/SNPCheckV3/snpcheck.htm) and RaW Probe Software (RaW-Soft v.015β) respectively. A standard probe set for 4 Q-fragments, 2 D-fragments, 15 genomic references, the X-chromosome and Y-chromosome was available for validation and analysis (SALSA P300-A2, MRC-Holland).

MLPA Reactions

The experiments were conducted according to the general instructions. Each experiment included eight reactions in which 5µl genomic DNA (10ng/µl) from each of seven samples and from a negative control was used (Supplemental Information).

Ethics Statement

Blood samples of patients were obtained and stored in the course of treatment following the Dutch Code for the proper secondary use of human samples. All patients provided verbal informed consent for molecular diagnostics of two genes (*PRKCSH* and *SEC63*) associated

with ADPLD during management. Participant's verbal consent was written in his/her medical record by the physician. This consent procedure and use of DNA for research was reviewed and approved by the regional ethics review board "Commissie Mensgebonden Onderzoek (CMO) regio Arnhem-Nijmegen" (CMO 2012/317).

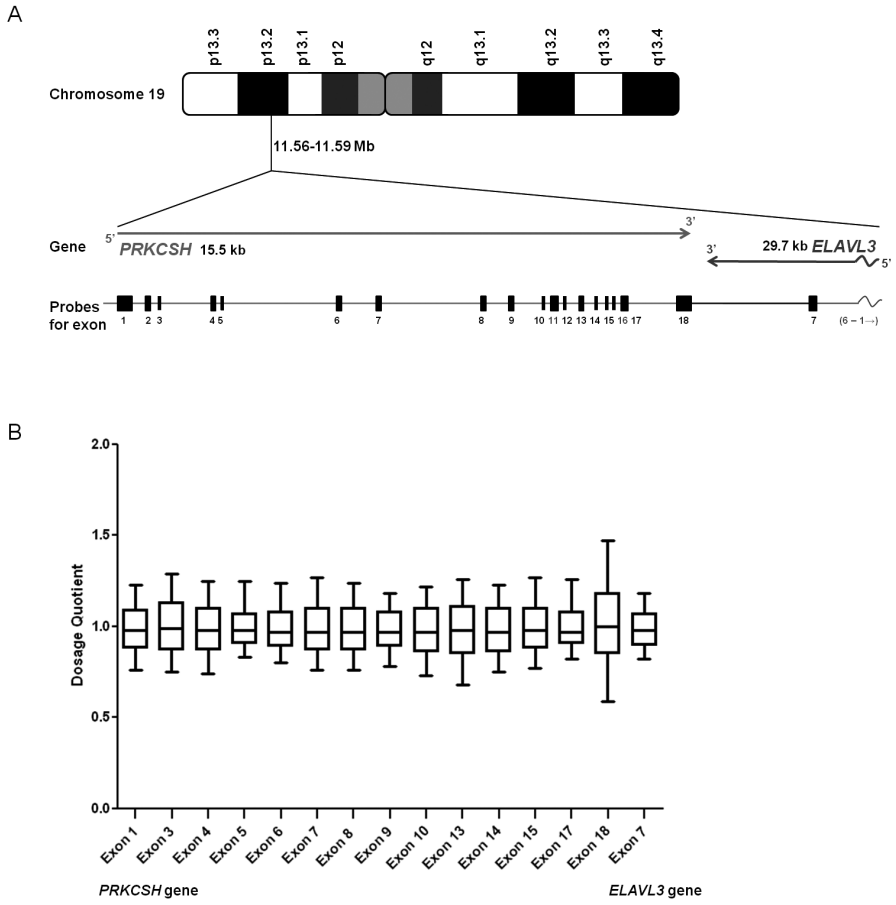

Figure 1. MLPA probe design and results. Each reaction consisted of 16 synthetic *PRKCSH* probes and 1 synthetic probe for exon 7 of the *ELAVL3* gene. Rationale of probe design. The direction of the open reading frame (ORF) of *PRKCSH* (5'→3') is in opposite direction of the ORF of the *ELAVL3* gene (3'←5'). The stopcodon of *ELAVL3* is located in exon 7. Large or single exon deletion of *ELAVL3* exon 7 may result in a transcriptional read-through into *PRKCSH* with subsequent promotor hypermethylation and reduced hepatocystin expression. DNA fragment analysis in genomic DNA samples from 56 mutation-negative ADPLD patients demonstrated no CNA for *PRKCSH* nor deletion of the probes for *ELAVL3*. Box plots present the performance of each probe set by indicating mean dosage quotient with range (minimum-maximum) and standard deviation.

RESULTS

MLPA Performance

Two genomic DNA samples of sufficient quality were included for the analysis as negative and reproducibility controls. Repetitive test runs with controls and genomic DNA samples were conducted for optimization of the experiments. The probe design for 14 exons of *PRKCSH* and *ELAVL3* exon 7 yield sufficient coverage.

The dosage quotient (DQ) is related to the amount of fluorescence detected within DNA fragment analysis. The ratio of the peak height of the synthetic or reference probe over the average of at least 12 reference (control) probes of a sample was determined. Signal intensity was absent for *PRKCSH* exons 2 and 12 in controls and all patient samples. These exons were not included for subsequent MLPA analyses. The reference probe set was within a normal DQ range (0.85-1.15). These results were used to investigate copy number aberrations (CNAs) in genomic DNA.

The genomic DNA samples in our study were within normal range of 0.68-1.18 for 14 *PRKCSH* probes. The probe for the final *PRKCSH* exon 18 had a mean DQ of 1.00 with a wider range (0.59-1.47). MLPA analysis on 56 mutation-negative patients showed no CNA in *PRKCSH* (Figure 1B). In addition, we investigated whether germline deletions were present in two mutation-negative ADPLD patients from *PRKCSH*-affected family. In both patients no exon deletions were identified (Figure S1, Table S2).

DISCUSSION

This study is the first report that describes the diagnostic properties of MLPA for *PRKCSH* in a cohort of patients with severe polycystic liver disease. Current molecular diagnostic testing of clinically diagnosed ADPLD patients consists of direct Sanger sequencing of the *PRKCSH* and *SEC63* genes. We hypothesized that large deletions and genomic rearrangements of *PRKCSH* may account for a proportion of mutation-negative ADPLD cases. Identification of somatic deletions of the *PRKCSH* gene suggests that this gene may be predisposed to deletions.[13] ADPLD is clinically and genetically heterogeneous and germline deletions may explain the majority of mutation-negative patients (~80%).[10, 12] Our findings revealed no large germline deletions or duplications in the *PRKCSH* gene from ADPLD patients without pathogenic variants in *PRKCSH* and *SEC63*. This negates a substantial role for MLPA in the diagnostic setting algorithm of ADPLD (Table 1).

These findings contrast studies that demonstrate exon deletions in ARPKD[19-21] and ADPKD[22-26]. About ~90% of ARPKD patients harbor a pathogenic variant in the *PKHD1* gene. The same is true for ADPKD patients with *PKD1* or *PKD2* mutations.[27, 28] There is a wide spectrum of genetic variation in ARPKD and ADPKD as nonsense, splice site, missense mutations, small deletions/insertions, but also extensive genomic rearrangements may be present. Previously, large deletions were detected in *PKD1* (n=4) and *PKHD1* (n=3) by long-range PCR and quantitative real-time PCR techniques.[19, 26] Recently, MLPA identified gross rearrangements of both ADPKD genes in 4% of the ADPKD-CRISP cohort.[22] Other studies found genomic deletions in 0-2.4% of *PKD1* carriers.[23, 25] Large deletions seem to be more frequent in ADPKD

Table 1. Overview of *PRKCSH* germline mutations. Deletions and duplications in *PRKCSH* germline DNA (NM_002743.2). Small deletions/ duplications are defined as ≤20 deleted/inserted base pairs (GRCh37; hg19)

	Germline mutation (*c.DNA*)	**Predicted protein effect**	**Study group/ reference**
Small deletions	*c.368delA*	p.E123GfsX130	Waanders et al.[10]
	c.374_375delAG	p.E125VfsX145	Drenth et al.[34]
	c.668delA	p.D223VfsX231	Waanders et al.[10]
	c.684-3_684delCAGG	p.V229X	Waanders et al.[10]
	c.1336delC	p.G447AfsX463	Waanders et al.[10]
	c.1440+2_1440+3delTG	p.T448CfsX457	Li et al.[7]
Small duplications	*c.76_79+4dupACCAGTGA*	p.N27SfsX117	Waanders et al.[12]
	c.215dupA	p.N72KfsX81	Li et al.[7]
	c.353dupA	p.K119EfsX122	Waanders et al.[10]
	c.1170dupC	p.p.I391HfsX401	Li et al.[7]
Gross deletions	-	-	This study
Gross duplications	-	-	This study

compared to ARPKD. Only 2 out of 16 ARPKD probands harbored a large *PKHD1* deletion.[21] In addition, there were no *PKHD1* rearrangements in 39 Dutch/Caucasian families.[20] These results suggest that large deletions are rare in polycystic kidney diseases.[25] Gross genomic rearrangements of *PKD1* are associated with deletion of the adjacent *TSC2* gene and referred as the TSC2/PKD1 contiguous syndrome (PKDTS).[22-24] In line, we designed additional probes for exon 7 of *ELAVL3*, a gene located 361bp upstream in antisense direction of *PRKCSH*. The deletion of this exon could result in transcriptional read-though (Figure 1A). Consequences of this mechanism may be *PRKCSH* promoter methylation and gene silencing. This type of epigenetic inactivation has been suggested for development of polycystic kidneys.[29] This mechanism was also found to be involved in Lynch syndrome. Patients presenting with Lynch syndrome, but no mutation in causative gene *MSH2*, were found to have a deletion of the last exon (including the stop codon) of upstream gene *EPCAM*. Formation of an extended *EPCAM* transcript inactivates *MSH2* due to promoter methylation.[30]

In tumorgenesis and polycystic kidney and liver disease, LOH is a common mechanism. Somatic *PKD1* deletions are demonstrated in cystic kidney and liver tissues from ADPKD patients.[31] Indeed, the 'second hit hypothesis' was confirmed for *PRKCSH* and *SEC63* on the somatic level in ADPLD.[13, 32] For *PRKCSH*, the mutation rate in genomic DNA (15%) and LOH regions in somatic DNA (76%) are the highest.[10, 13] Detection of somatic deletions by MLPA is challenging, but suggested to be applicable.[22]

Surprisingly, we have not identified germline deletions nor duplications in a large cohort of mutation-negative ADPLD patients. No unstable genomic regions which may be prone to rearrangements in the *PRKCSH* gene are identified, as such is known for duplicated regions

in the large *PKD1* gene.[33] In addition, we expected to identify an exon deletion in two mutation-negative ADPLD patients coming from a family with a known *PRKCSH* mutation.

Germline deletions in two other ADPLD genes, *SEC63* and *LRP5*, are not ruled out by this study. The frequency of small deletions in the *SEC63* gene (n=4; 21%) or in the *PRKCSH* gene (n=7; 26%) are almost similar.[10] Currently, only *LRP5* missense mutations are associated with ADPLD. Therefore, the possibility of large germline deletions in the *SEC63* or *LRP5* gene is probably minimal or absent. We hypothesize that in these affected individuals and other patients with a clinical diagnosis of ADPLD harbor mutations in yet unidentified gene(s) associated with hepatic cystogenesis.

In conclusion, no large deletions and duplications are present in the *PRKCSH* gene in germline DNA from mutation-negative ADPLD patients. This suggests that a fourth as yet unidentified locus is involved in ADPLD development.

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SUPPLEMENTARY METHODS

MLPA Reactions

DNA was denaturated for 5 minutes at 98°C in a thermocycler (iCycler, BioRad Laboratories Inc, Hercules, California, USA). After cooling to room temperature the samples were subjected to a hybridization step by adding 1.5µl probe mix and 1.5µl SALSA/MLPA buffer. The probe mix included a solution of (2/3) the SALSA reference probe mix and (1/3) the synthetic basic probe mix (1µM oligo solution LPO and RPO). The final mix was denaturated for 1 minute at 95°C and hybridized during 16 hours at 60°C. After cooling the samples to 54°C, the ligation reactions were processed for 15 minutes at 54°C by adding 32µl Ligase-65 master mix to each sample. An additional incubation for 5 minutes at 98°C inactivated the ligase enzyme. Next, a polymerase mix was added to conduct a PCR amplification with 35 cycles (30 seconds; 95°C, 60°C, 72°C) and finally 20 minutes at 72°C.

MLPA Fragment Analysis

PCR products were analyzed for quality on a 2% agarose gel. For fragment analysis PCR products were diluted 1:9 with Liz-Formamide mix containing 1/44 500LIZ Size Standard (Applied Biosystems). DNA fragment separation was performed by capillary electrophoresis on an ABI-3730 (Applied Biosystems), and data of peak height and DQ were analyzed using Gene mapper software (v.1.01 Applied Biosystems) according to manufacturers' instructions. Ligation quality (D-fragment) and DNA quality (Q-fragments) were assessed for the DNA fragment analysis of each reaction.

Calculations

Calculated dosage quotient (DQ) is related to the amount of fluorescence detected within DNA fragment analysis. The ratio of the peak height of the synthetic or reference probe over the average of at least 12 reference (control) probes of a sample was determined. Secondly, the value of this ratio was divided by the average of the corresponding probe of all samples. Reference DQ-values were provided by MRC-Holland to identify potential deletions or duplications. A heterozygous deletion is defined as a DQ between 0.35 and 0.65, and a heterozygous duplication when DQ is between 1.35 and 1.55. A normal copy number status is a DQ reference value between 0.85 and 1.15 according to manufacturers' instructions (MRC Holland).

SUPPLEMENTARY FIGURE

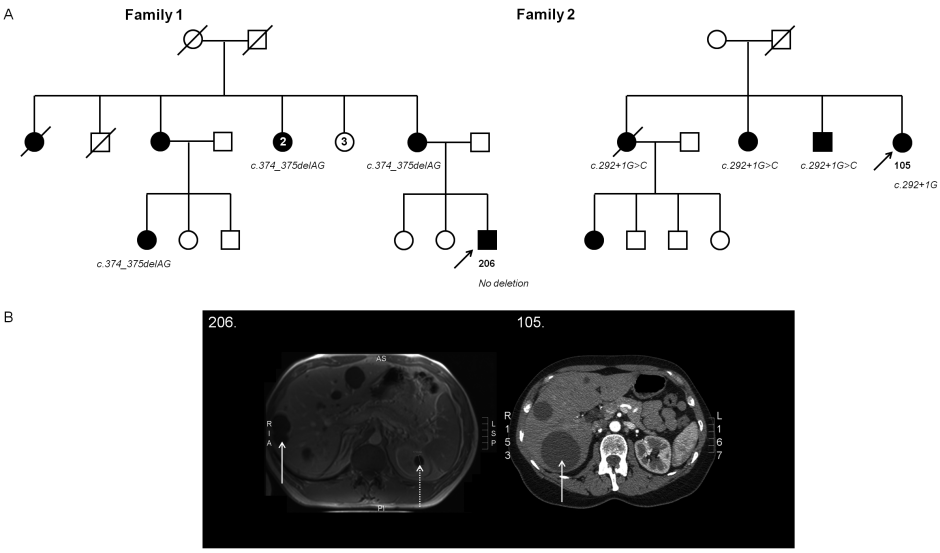

Figure S1. Two Dutch ADPLD families with a known *PRKCSH* mutation. Two families presented clear autosomal dominantly inherited ADPLD. Except 1 patient each, all affected individuals from both families presented a known *PRKCSH* mutation by diagnostic Sanger sequencing.[6, 34] Family 1 harboured *c.374_375delAG* (p.Glu125fs*21) and ADPLD patients from family 2 carried *c.292+1G>C* (p.D98AfsX100). Pedigree of ADPLD family 1 and 2 in which the patients (206 and 105) without *PRKCSH* mutation are indicated by an arrow. Clinical liver and kidney features of mutation-negative ADPLD patients by abdominal MRI or CT scanning. Patient 206 has a multiple cysts scattered throughout the liver (white arrow) and several small renal cysts (dotted white arrow). A large hepatic cyst (white arrow) caused major complaints in patient 105 with clinical diagnosis of ADPLD.

Table S1. MLPA synthetic probe design for screening deletions/duplications in the *PRKCSH* gene.

Exon	Left hybridising probe (5'-3')	Right hybridising probe (5'-3')	Length
PRKCSH-exon01	TGCGCCGCCATTAAGAGCTAGCCCTCAGAACTATC	Phos-CTCTCTAAGGTTTCACAGTCCTGCCCAAGATGGCC	35
PRKCSH-exon02	TCCTCCCACAGAACCCGTTTCGGGCCTCAGAGCGTCTGGT-GAGATGCTG	Phos-GTGAGATGCTGTTGCCGCTGCTGCTGCTGCTACCCATGT-GCTGGGCCGT	49
PRKCSH-exon03	TCACCTGCCTGGACGGTTCGGCCACCATCCCATTTGATCAG-GTCAAC	Phos-GATGACTATTGCGACTGCAAAGATGGCTCTGACGAGC-CAGGTGAGCC	47
PRKCSH-exon04	CCCTCCCTTCTTCCTCACAGG	Phos-CACGGCTGCCTGTCCTAATGG	21
PRKCSH-exon05	gggaggaggcactgccaggtctgatcttg	Phos-GCTTCTGCCTCTGCCACAGACTGCTGCGA	29
PRKCSH-exon06	GAAGGGCCGTAAGGAGAGAGAGTCCCTGCAGCAGATGGC	Phos-CGAGGTCACCCGCGAAGGGTTCCGTCTGAAGAAGATCCT	39
PRKCSH-exon07	GGCAGAACAGAGGAGAGCTGGTCTCTTGCCTTCTGCCCACC-CAGAAAAAGCTCATTGAG	Phos-CTACAGGCTGGGAAGAAGTCTCTGGAAGACCAGGTGGA-GATGC TGCGGACAGTGAAGGA	59
PRKCSH-exon08	ggcaggggtgacagaggtggcttctttacagagcagctggctgctgccaaggc-ccaacag	Phos-GAGCAGGAGCTGGCGGCTGATGCCTTCAAGGAGCTGGAT-GATGACATG GACGGGACGTGAG	51
PRKCSH-exon09	GTGACTGAGCTGCAGACTCACCCGGAG	Phos-CTGGACACAGATGGGGATGGGGCGTTG	27
PRKCSH-exon10	TAGATCTTGACACCACCCCCAACACACACAGGCCCTC	Phos-CTCAGTGGGGACACACAGACAGACGCCACCTCTTTCT	37
PRKCSH-exon12	CTACGACGAGCAGACGCAGGCCTTCATCGATGGT-GAGGGTGGGCGGGGGCCAGGCTC	Phos-CTCGGGTGGGCCCAGCGTTTCCTGCCGTGGTGGCACAG-GTCGAGGGAAG ATCCTGAG	49
PRKCSH-exon13	GCTGCCCAGGAGGCCCGCAACAAGTTCGAGGAGGC-CGAGCGGTCGCTGAAGGACATG	Phos-GAGGAGTCCATCAGGTAGCGGGGGCTGAGGAGCGGG-GACACCTGTCC CACAGCGACT	50
PRKCSH-exon14	TGACTTTGGCCCCAACGGGGAGTTTGCTTAC	Phos-CTGTACAGCCAGTGCTACGAGCTCACCACCA	31
PRKCSH-exon15	CGGTCCTCCACAGATACGTCTACCGCCTCTGCCCCTTCAAG	Phos-CTTGTCTCGCAGAAACCCAAACTCGGGGGCTCTCCCAC-CAG	41
PRKCSH-exon17	CCAGGTGCGCCTCCTGTGCGGGAAAGAGACCATGGTGAC-CAGCAC	Phos-CACAGAGCCCAGTCGCTGCGAGTACCTCATGGAGCTGAT-GACGCC	45
PRKCSH-exon18	TGCCCCAGAACCTCAAGAAGGCATGAAGCCAGCCCCT-GCAGTGCCGTCCACCCGCCCCTCTGG	Phos-GCCTGCCTGTGGCTCTGTTGCCCTCCTCTGTGGCGGCAG-GACCTTTG TGGGGCTTCGTGCCCT	63
ELAVL3-exon07	GTGCATCTTCGTGTACAACCTGTCACCGGAGGCAGACGAGA-GCGTGCTGTG	Phos-GCAGCTGTTCGGGCCTTTTGGGGCAGTCACCAACGTCAA-GGTCATCCGTGA	51

Table S2. Baseline characteristics of two mutation-negative ADPLD patients belonging to a *PRKCSH*-positive family.

	ADPLD patient 206: Family 1	**ADPLD patient 105: Family 2**
Mutation status affected family members (*c.DNA* and predicted protein effect)	*c.374_375delAG* (p.Glu125fs*21)	*c.292+1G>C* (p.D98AfsX100)
Nature of mutation; location	Deletion; exon 6	Splice site substitution; IVS4
Protein domain	-	LDLa
Sex	Male	Female
Age at diagnosis (MRI/CT scan)	39	49
Clinical symptoms	Abdominal swelling	Abdominal pain, fatigue, back pain, vomiting
Treatment strategy	-	Anti-reflux therapy

CHAPTER 5

SOMATIC HITS IN POLYCYSTIC LIVER DISEASES

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ABSTRACT

Polycystic liver disease (PLD) encompasses a number of disorders with the development of multiple cysts distributed throughout the liver either focally or equally. Hepatic cysts are fluid-filled cavities lined by benign epithelium. PLD is the major phenotype of isolated polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD).

The molecular principles in carcinogenesis indicate that there is an accumulation of multiple (somatic) mutations. This concept assumes that presence of a germline mutation ('first hit') in an inherited disorder requires a 'second hit' at the somatic level for cyst development to occur. The second hit is the rate-limiting step and results in somatic inactivation of the normal allele.

Studies have identified secondary, somatic hits in human liver cyst tissues in PCLD and ADPKD. Inactivation of both copies in PLD is demonstrated through somatic mutations or loss of heterozygosity (LOH). The frequency of somatic mutations varies between genes and genomic disorders. Genetic studies detected LOH in 9% and somatic mutations in 8-29% in ADPKD derived hepatic cysts. In PCLD, almost ~80% of hepatic cysts from *PRKCSH* carriers had completely lost the *PRKCSH* gene.

There is important clinical heterogeneity among PLD patients. Differences in phenotypical expression may be explained by age, gender and environment, but also modifier genes or inactivating somatic events may play key roles. This review will give an overview of the data gained from genetic studies in liver cyst tissues from PCLD and ADPKD patients in relation to the clinical manifestations.

Keywords

Second hit hypothesis; Somatic mutation; LOH; Loss of function; PLD; PCLD; ADPKD

INTRODUCTION

Polycystic liver disease (PLD) comprises a group of diverse congenital disorders that have presence of multiple hepatic cysts in common. There are 2 major conditions that possess this benign phenotype, isolated polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD).[1, 2] Both Mendelian disorders are autosomal dominantly inherited and share similar liver features.[3] PCLD is characterized exclusively by presence of hepatic cysts, and polycystic livers are the most common extrarenal manifestation in ADPKD.[4] Development of polycystic kidneys is a key feature in ADPKD, but also cardiovascular manifestations, intracranial aneurysms, pancreatic cysts and renal complications with end-stage renal disease may be present.[1, 3]

The origin of hepatic cystogenesis probably starts in early embryological phase with biliary tree development. The formation of the ductal plate is needed for development of healthy bile ducts. In genetic disorders leading up to PLD this process is compromised, hence the term ductal plate malformation.[5] It is likely that the nidus needed for cyst formation (microcysts) is already present in childhood of germline carriers. These microcysts are undetectable by routine radiological methods, but may develop later in life.[4]

Following Knudson 'second-hit' hypothesis, it was proposed that cysts arise as a result of a second mutational event. Patients carrying a germline mutation (the 'first hit' in a PLD gene) are prone for cyst development. However, the single mutated allele does not necessarily lead to hepatic cysts, but a second somatic event is required for individual cysts to develop.[6]

In this short review we present a comprehensive overview of somatic mutations and genetic mechanisms that have been associated with hepatic cystogenesis in PLD.

GENETIC BACKGROUND

PCLD and ADPKD are distinct disorders and associated with germline mutations in separate genes. About ~25% of PCLD patients carry a bonafide gene mutation in the *PRKCSH* gene or *SEC63* gene.[7-10] Both protein products, hepatocystin and Sec63p, are involved in protein folding, quality control and transduction in the endoplasmic reticulum.[11] Almost all ADPKD patients carry a pathogenic germline *PKD1* gene (~85%) or *PKD2* gene (~15%) mutation.[12-14] The corresponding protein products polycystin-1 (PC-1) and polycystin-2 (PC-2) function as a mechanosensory receptor-channel complex at the primary cilium for calcium influx.[1]

The primary cilium is an extending organelle at the plasma membrane of the bile duct epithelium cell (cholangiocyte). Pathofysiological processes are functionally engaged with this structure by detection of the luminal flow. Defective polycystin expression contributes to decreased intracellular calcium and increased cAMP levels.[15] The combination of these abovementioned mechanisms and overexpression of PKA and Epac cause cholangiocyste hyperproliferation through the mitogen-activated protein kinase/extracellularly regulated kinase (MAPK/ERK) pathway and cyst fluid hypersecretion in PLD.[16] Current somatostatin analogue treatment affects secretin-mediated pathways (Figure 1).[17]

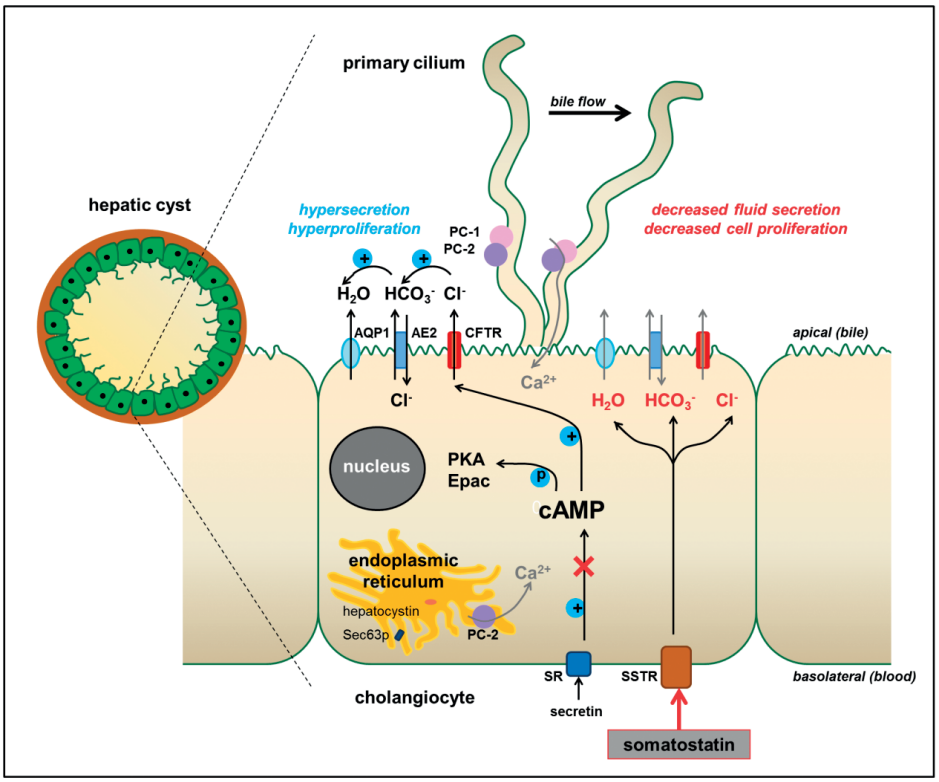

Figure 1. Somatostatin treatment decreases cholangiocyte hyperproliferation and fluid hypersecretion in PLD. Hepatic cysts are delineated by bile duct epithelium cells. The primary cilium at the plasma membrane is continuously exposed to luminal bile flow. Mechanical flow rate is sensed by bending the organelle. Under healthy conditions this stimulates calcium signaling and inhibits forskolin-stimulated cAMP signaling intracellularly.

PC-2 co-localizes with PC-1, and PC-2 is a calcium channel expressed at the endoplasmatic reticulum. Cholangiocytes possess numerous transporters and exchangers. Basolateral secretin stimulates intracellular cAMP signaling and bicarbonate fluid rich secretion by activation of apical CFTR chloride channel. Subsequently, secreted bicarbonate drives passive AQP1-mediated water transport to the extracellular compartment.

This figure illustrates a defective molecular mechanism in PLD. Decreased intracellular calcium and accumulation of second messenger cAMP contributes to hepatic cystogenesis.[15] Phosphorylation of PKA and Epac are associated with increased MAPK/ERK signaling in PLD. In response to this activated pathway fluid secretion and cell proliferation is facilitated.[16]

Somatostatin acts via SSTR-2 somatostatin receptors to increase cGMP which inhibits secretin-mediated cAMP synthesis. Secretin-stimulated chloride, bicarbonate and water secretion are inhibited and absorption is induced by somatostatin analogues (in red).

TUMORGENESIS

General Principle in Tumorgenesis

In 1953 Nordling was the first to propose the multi-mutation hypothesis for the origin of cancer. His theory stated that accumulation of DNA mutations in a cell as a consequence of

Table 1. Somatic mutations with the predicted effect on protein level in liver cyst tissue from PLD patients. Types of somatic hits included 5 substitutions, 3 deletions and 1 insertion mutation at the similar gene locus (GRCh37-hg19). These mutations resulted in pathogenic missense changes or truncated protein products. *TranscriptID. *PRKCSH* (NM_002743.2); *PKD1* (L33243.1); *PKD2* (NM_000297.2)

PLD sampleID.	Germline mutation(s)*	Predicted protein effect	Somatic mutation*	Predicted protein effect	Ref.
ADPKD1 JHU415	*PKD1 c.12378C>G*	p.Tyr4126X	*PKD1 c.12551insGC*	p.His4185Argfs*13	[33]
ADPKD1 JHU452	*PKD1 c.7165T>C*	-	*PKD1 c.8900C>G*	p.Ser2967X	[33]
	PKD1 c.9047A>G	p.Gln3016Arg	*PKD1 c.10050+2del20*	aberrant splicing	
			PKD1 c.8558T>C	p.Phe2853Ser	
			PKD1 c.7567G>T	p.Glu2523X	
			PKD1 c.8733del16	p.Asp2912Argfs*77	
ADPKD2 UT1500	*PKD2 c.2152insA*	p.Asn720Lysfs*5	*PKD2* c.710-8del19	aberrant splicing	[30]
PCLD patient 1	*PRKCSH c.1341-2A>G*	aberrant splicing	*PRKCSH c.224T>C*	p.Phe75Ser	[34]
			PRKCSH c.1499G>A	p.Cys500Tyr	

normal cell proliferation, environmental exposure and increasing age results in carcinogenesis.[18] Clinical and epidemiological observations prompted Knudson to reformulate this hypothesis with the example of dominantly inherited and acquired retinoblastoma.[19] He proposed a 'second hit' hypothesis which implicates that a second mutation is required in addition to a 'first hit' for tumor development.

Since then, several studies in common malignant diseases, particularly those caused by tumor suppressor genes such as ovarian and colon cancer, have produced data consistent with the mutational origin of cancer. This concept is refined and proof is provided that occurrence of 2 (somatic) 'hits' may be sufficient for tumor development in sporadic malignant diseases.[20, 21] The result from both mechanisms is biallelic inactivation through loss of the wild-type copy. Second hits are usually small deletions or insertions resulting in a truncated protein, but large deletions at the gene locus with loss of heterozygosity (LOH) are also seen.[20-24]

Identification of Somatic Inactivation in ADPKD and PCLD

Subsequent to the identification of the first causative *PKD1* gene for ADPKD, researchers have speculated about presence of somatic mutations that would lead to the inactivation of the second *PKD1* allele.[12] In order to address this issue, methods have been developed to isolate cyst-lining epithelial cells to access DNA from single cysts. These studies confirmed the second hit hypothesis in renal cyst epithelium from *PKD1* affected patients.[23, 25, 26]

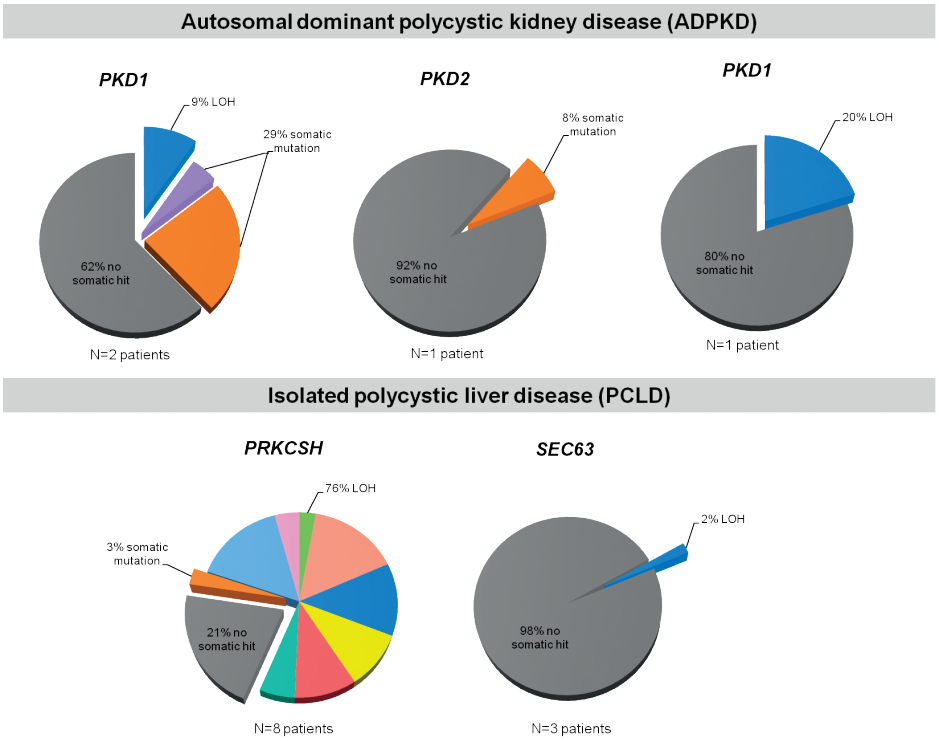

Figure 2. An overview of reported somatic hits in liver cyst tissues from PLD patients. Identification of LOH or somatic mutations in *PKD1* or *PKD2* in ADPKD[28, 30, 33], and in *PRKCSH* or *SEC63* in PCLD[34, 35] presented by a colored part per patient. Grey pieces represent no detected somatic hits in hepatic cysts.

Kidney

Genetic analyses using microsatellite markers established loss of the healthy *PKD1* allele in 4-24% of renal cysts from *PKD1* affected ADPKD patients. Somatic mutations were reported in 17% of renal cysts from a *PKD1* mutation carrier.[23, 25-28] Likewise, renal cysts from ADPKD patients harboring a *PKD2* germline mutation had LOH at the *PKD2* locus with a frequency of 0-12% and (milder) somatic *PKD2* mutations were present in 15-64% of renal cysts.[29-32] In addition, these analyses showed that the majority of single renal cysts in ADPKD presented epithelial cell populations derived from 1 cell. These experiments fueled the idea that cysts consist of a monoclonal cell population build from a single cell affected by 2 hits.[23, 25]

Liver

Analysis of hepatic tissues (21 cysts) from 2 ADPKD patients that were *PKD1* mutation carriers showed that the other allele was lost in both cases because of LOH (9%) or somatic mutations (29%) (Figure 2; Table 2).[33] Another study in an ADPKD patient unlinked to *PKD1*, the healthy *PKD1* allele in hepatic cyst epithelium was lost in 20% because of LOH.[28] The situation was different in a *PKD2* carrier. Somatic hits were detected in 1/13 (8%) cysts using classic intragenic and microsatellite marker analyses.[30] By contrast, direct Sanger sequencing

Table 2. Overview of the number of hepatic cyst tissues per PLD patient for evaluation of somatic hits. An extended number of hepatic cysts in several patients have been analyzed in PCLD compared to ADPKD; n=71 cysts and n=52 cysts in *PRKCSH* and *SEC63* affected individuals respectively. *TranscriptID. *PRKCSH* (NM_002743.2); *PKD1* (L33243.1); *PKD2* (NM_000297.2)

Study group	Phenotype	Patients germline mutation(s)*	Hepatic cysts (n)	Ref.
Watnick et al. 1998	ADPKD1	JHU415. *PKD1 c.12378C>G*	12	[33]
	ADPKD1	JHU452. *PKD1 c.7165T>C* and *PKD1 c.9047A>G*	9	
Pei et al. 1999	ADPKD2	UT1500. *PKD2 c.2152insA*	13	[30]
Badenas et al. 2000	ADPKD1	3. Germline mutation not available	15	[28]
Janssen et al. 2011	PCLD	1. *PRKCSH c.1341-2A>G*	14	[34]
	PCLD	2. *PRKCSH c.1341-2A>G*	12	
	PCLD	3. *PRKCSH c.1341-2A>G*	9	
	PCLD	4. *PRKCSH c.1341-2A>G*	9	
	PCLD	5. *PRKCSH c.292+1G>C*	5	
	PCLD	6. *PRKCSH c.292+1G>C*	13	
	PCLD	7. *PRKCSH c.292+1G>C*	7	
	PCLD	8. *PRKCSH c.1341-2A>G*	2	
Janssen et al. 2012	PCLD	1. *SEC63c.1703_1705delAAG*	34	[35]
	PCLD	2. *SEC63 c.1703_1705delAAG*	4	
	PCLD	3. *SEC63 c.958G>T*	14	

studies at the somatic level in 71 cysts from PCLD patients (*PRKCSH* carriers) detected a LOH incidence of 76% at the *PRKCSH* locus.[34] This is different from *SEC63* mutation carriers where *SEC63* LOH was present in 2%.[35]

Identical to the situation in common tumors, the type of somatic mutations in PLD can be substitutions, small deletions or insertions leading to a missense change or premature stopmutation (Table 1).[30, 33, 34] PCLD patients harboring a *PRKCSH* germline mutation are highly at risk for somatic mutational inactivation. Loss of the PRKCSH protein influences progression of hepatic cystogenesis. Therefore, it is possible that *PRKCSH* acts as a tumor suppressor gene.

Somatic Inactivation in Sporadic Hepatic Cysts

Sporadic cysts may arise as an incidental finding or as an acquired hepatic cyst from a traumatic or an infectious condition. Simple hepatic cysts occur in about ~10% of the general population and are frequently asymptomatic.[6] However, sporadic cysts may increase in volume and cause pressure on surrounding organs.

Germline mutations are required for cyst formation in congenital PLD, but are usually absent in individuals with a sporadic cyst. It is likely that loss of PLD genes occurs through

somatic inactivation. Depending on the target tissue, loss of ADPKD alleles may lead to renal as well as hepatic cysts.[30, 33]. This mechanism has not (yet) been identified in PCLD patients.

A loss-of-function model could be hypothesized as a common molecular mechanism in PLD. Different mutation types, deletions or LOH of a region including a PLD gene has been stated, but LOH may be also the result of errors during meiosis I or II. In acquired uniparental disomy an individual received 2 chromosomes from 1 parent. This abnormal normal haplotype leads to disease in case this allele is non-functional. The consequence of this gene conversion is that the patient carries a copy number neutral LOH. For example, LOH in combination with these mechanisms are detected in other benign tumors such as neurofibromatosis, but also in malignancy.[22, 24]

Protein Expression Levels in Liver Cyst Tissue

Immunohistochemistry analyses of gene products may indicate the presence or the expression level of proteins. Patients harboring a truncating heterozygous germline mutation usually have a second, wild-type allele. Following the second hit hypothesis in PLD, this wild-type allele will be lost and no protein expression will be detected by staining experiments. Indeed, studies of liver cyst tissues from patients with a germline *PRKCSH* mutation demonstrated that the PRKCSH protein was absent in cyst epithelium, but Sec63p was expressed.[34, 36] Vice versa, the cyst that harbored somatic LOH of *SEC63* showed reduced expression of the Sec63p, but positive PRKCSH staining.[35] These findings suggest that both PCLD protein products do not interact.

On the contrary, genetic studies in human liver cyst tissues from *PKD2* affected patients [29, 32] found that the majority represented polycystin-2 expression and equal polycystin-1 expression.[37] Molecular studies showed that polycystins interact by coiled-coil domains to form multimeric complexes. Interaction studies in ADPKD showed that polycystin-1 is a regulator of polycystin-2 activity in liver and kidneys.[38] This argues that *PKD* mutation carriers share the predominant phenotype of polycystic kidneys.

Trans-Heterozygous Model

The first hypothesis of a trans-heterozygous model was confirmed with experiments in renal cysts in a *PKD1* affected ADPKD family. This study revealed that *PKD1* germline derived cysts could have somatic *PKD2* mutations.[27] Next, clonal *PKD1* somatic hits were discovered in renal cysts from *PKD2* carrier.[31] These observations demonstrate an alternative pathogenic mechanism for cyst formation. To date, the trans-heterozygous model has been excluded in PCLD. Co-existence of second hits at the *PKD1* or *PKD2* locus in PCLD may compromise the functional network in PLD.[39, 40]

Clinical Heterogeneity in Polycystic Liver Disease

The liver phenotype of inherited PLD may range from a single or few cysts to an advanced polycystic liver with numerous cysts. Usually, several liver segments remain unaffected and the liver function is preserved, even in severe PLD.[2, 3]

Individual and inter-patient differences of cyst size, localization and growth suggest that other factors are involved in the pathogenesis. The type of somatic mutations may explain to some extent the individual variability. For example, the number and type of somatic mutation in the tumor suppressor gene *APC* has important implications on the protein function resulting in growth advantages of the cell.[41]

As indicated, the diversity of clinical presentation in families is high. The penetration of the disease has been estimated at ~80% and even if the disease become penetrant, its variability is high.[10] Some affected family members may develop early and severe disease, while others only develop minor symptoms. This may occur among those sharing identical germline mutations. Although family history is frequently negative in PLD, an asymptomatic carrier can transmit the disease to offspring who may become affected.

PLD patients may be asymptomatic for many years. The number and size of hepatic cysts increase progressively by the age of 30 resulting in advanced disease in patients in their fifth decade.[4] This variation reminds us of the situation in retinoblastoma. An early-onset of disease could be explained by the high rate of a second hits in embryonic cells.[19] The mutation rate of the somatic hit determines the progression of the disease. It is likely that in PLD these somatic hits are the rate-limiting step for PLD development.

DISEASE MODEL

Examination of the second hit model has afforded us insight in the molecular mechanisms of PLD. Genome-wide copy number and (copy number neutral) LOH regions from liver cyst tissues are of high interest, because these investigations may guide us to putative candidate genes for hepatic cyst formation. Next, LOH regions in cysts of target tissue (liver or kidney) may indicate modifiers or novel genes at the germline level which contribute to monoclonal hyperproliferation of cholangiocytes in congenital and sporadic cysts.

It is likely that more disrupted gene products are involved in PLD, because multiple pathways and modifiers affecting cystogenesis are identified. These reasons might explain similarities and differences in clinical presentation. For example, the polycystins are functionally part of similar signaling pathways. A recent study in animal models provided functional evidence for presence of trans-heterozygous mechanisms in PLD. Reduced hepatocystin or Sec63p expression leads to hepatic and renal cystogenesis, but also affects the functional polycystin complex. These data provide evidence for a PLD protein network responsible for cystogenesis. Perturbation of polycystin-1 levels regulate the disease severity in a dosage-related fashion.[39]

CONCLUDING REMARKS

The nature of individual cyst formation has been associated with genetic mechanisms that are common in tumorgenesis. For initiation and promotion of a malignant or benign tumor, more than 1 mutation is required.[6, 19] In case of a hepatic cyst, both alleles of PLD-related genes are inactivated by a two-step process. The first hit is the inherited mutation present in the germline and the second hit is the onset of somatic events. Somatic hits can cover the

spectrum from missense mutations to LOH. A fine example is the situation in liver cyst tissue from *PRKCSH* mutation carriers with a high LOH rate of 76%.[34]

There is a high variability of liver phenotype in PLD patients that are of similar sex, age and share identical germline mutations. The focal character of intra-familial variability in PLD may be explained at least partially by genetic mechanisms at the somatic level. This paper lists the evidence supporting that inactivation of 2 PLD-gene copies underlies hepatic cyst formation.

Development of multiple fluid-filled cysts expands over time and affects the normal biliary architecture. These processes are influenced by age, gender (hormones), somatic hit rate and frequency, but probably also a significant dosage effect through accumulation of multiple mutations.[18, 39, 40]

The genetic threshold is low in inherited PLD because there exists already loss of 1 allele. On the other hand, somatic inactivation of 2 PLD alleles is also possible and in those cases isolated cysts arise. Both modes result in clonally proliferation, growth and individual cyst formation.

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CHAPTER 6

SOMATIC LOSS OF ALLELES FROM HEPATIC CYST EPITHELIUM IDENTIFIES CANDIDATE GENES IN POLYCYSTIC LIVER DISEASE

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ABSTRACT

Autosomal dominant polycystic liver disease (PCLD) is characterized by presence of multiple fluid-filled hepatic cysts. Germline mutations in *PRKCSH*, *SEC63* or *LRP5* underlie ~20% of PCLD cases and despite ubiquitous gene expression, the phenotype is restricted to the liver. We hypothesized that the second allele of these genes is affected by a somatic mutation in the cyst epithelium, as previously shown for *PRKCSH* cases. Identifying regions underlying somatic loss of heterozygosity (LOH) may also be a method to identify novel PCLD genes. Somatic LOH in cyst fluids can be detected by genome-wide SNP microarrays and may therefore directly point to the location of the germline mutation (the first hit).

We collected cyst fluid samples from patients with isolated hepatic cysts. Cyst fluid samples were obtained by aspiration sclerotherapy and subjected to centrifugation, cytokeratin-19 staining and fluorescent-activated cell sorting of cholangiocytes. Only cyst fluid with a lucid, clear content was eligible to process for further analysis and thus for a subset of 8 cases flow-sorted cells were lysed and DNA was amplified using whole-genome-amplification. Genome-wide SNP analysis followed to identify regions with somatic LOH after comparison with array results of genomic, blood-derived DNA.

Here, we identified large somatic LOH events in 2 out of 8 cases; one case harboring a heterozygous pathogenic *PRKCSH* mutation and a somatic LOH encompassing the *PRKCSH* gene; one other case presenting with a large LOH of chromosome 16p encompassing *PKD1* and several other genes. Surprisingly, we also identified several large germline events in some cases. In one patient we identified a 2.9Mb deletion as well as two large regions of homozygosity. We speculate that either the CNV itself or a homozygous point mutation may be involved in the PCLD phenotype of this patient. To address the latter, whole-exome sequencing of genomic DNA was applied.

Keywords

Polycystic liver disease; Cholangiocytes; Cell sorting; Loss of heterozygosity; Microarray; Candidate genes

INTRODUCTION

Isolated polycystic liver disease (PCLD) is an autosomal dominantly inherited disorder characterized by the progressive development of multiple biliary epithelial liver cysts.[1] A heterozygous germline mutation in the *PRKCSH*, *SEC63* or *LRP5* gene cause polycystic livers in about ~20% of PCLD patients.[2-6] The molecular mechanism of hepatic cystogenesis is recessive on the cellular level for *PRKCSH* and *SEC63* mutation carriers. Loss of the second, wild-type *PRKCSH* or *SEC63* allele in hepatic cyst epithelium may initiate cyst formation in these patients.[7, 8]

Approximately ~80% of PCLD patients harbor no *PRKCSH*, *SEC63* or *LRP5* mutations by extensive direct Sanger sequencing.[6, 9] PCLD is genetically heterogeneous and it is likely that at least one more gene is associated with hepatic cystogenesis.[6, 9] PCLD candidate genes may correspond to characteristics of known PCLD protein products such as hepatocystin and Sec63p located at the endoplasmic reticulum.[1] In addition, the Wnt signaling which is associated with *LRP5* and *DKK3*, but also other biological pathways may reveal new genes related to PCLD.[10]

Micro-dissected hepatic cyst epithelium from *PRKCSH* and *SEC63* carriers presented loss of heterozygosity (LOH).[7, 8] We hypothesized that this 'two-hit model' is a general principle for development of hepatic cysts in PCLD. Therefore, somatically deleted alleles in hepatic cyst epithelium may also occur for yet unknown PCLD genes. Loss of somatic allele copies in cyst fluid-derived epithelium cells, which can be detected by genome-wide SNP microarrays, may directly point to the location of the germline mutation. When validated in other cohorts, these data may lead to novel PCLD genes and new pathways associated with the pathogenesis of polycystic livers.

Another approach with the similar aim to identify candidate genes was successfully applied for related disorders such as autosomal dominant polycystic kidney disease (ADPKD). In order to elucidate molecular pathways involved in ADPKD, microarray studies of renal epithelial cells profiled expression levels of numerous candidate genes.[11-13] Aberrant *DKK3* expression directed to identification of 3 SNPs in the *DKK3* gene modifying disease progression in *PKD1* affected ADPKD patients.[13]

In this study we report a novel approach for the identification of PCLD genes. These findings may give clues for further genetic analysis.

MATERIALS AND METHODS

Patients and Samples

Symptomatic PCLD patients were referred to our hospital for aspiration sclerotherapy.[14] We collected 68 cyst fluid aspirates from 50 unrelated PCLD patients between January 2011 to December 2012. Only lucid, clear fluids could be used for further processing and thus 8 samples remained. Written informed consent from all subjects and ethical approval from the Medical Ethics Committee of the Radboudumc, The Netherlands was obtained.

SkChA-1 cells

For technical performance of immunofluorescence staining and cell counting assays the SkChA-1 cell line was used for test runs, protocol settings and as control sample for processing

cyst fluid samples.[15] SkChA-1 cells were cultured in DMEM supplemented with 10% FCS FCS (Life Technologies, Carlsbad CA).

Protocol Cholangiocyte Isolation

Processing cyst fluid

Cyst fluid aspirates were collected in a sterile environment and processed directly after the invasive procedure. The total amount of cyst fluid was equally divided in 50ml Falcon tubes for subsequent centrifugation. After 15 min 2,500rpm centrifugation, pellets were resuspended in a small aliquot of cyst fluid and transferred to 3ml tubes. Supernatant was manually eliminated. In case of total cyst fluid ≥200 ml, a maximum of 2 pellets derived from each 50ml cyst fluid, were combined in one 3ml tube.

Staining hepatic cyst cholangiocytes

For fixation 100µl medium A (Fix, Life Technologies) was added to the pellet and placed in the dark at room temperature for 20 minutes. A negative control (no medium A) and a positive control (SkChA-1 cells with medium A) was included during each run. Cells were washed with 2ml 0.5% BSA/PBS. Subsequently, after 5 minutes 500rpm centrifugation, supernatant was discharged. 100µl medium B (Perm, Life Technologies) with 1:100 diluted cholangiocyte marker anti-cytokeratin-19 (MU246-UC, mouse monoclonal, LOT MU2460706, Biogenex,Fremont CA)[16] in 0.5% BSA/PBS was added. The samples were placed in the dark at room temperature for 30 minutes. The washing step with 2ml 0.5% BSA/PBS, centrifugation and supernatant removal was repeated twice. After incubation of 100µl with 1:200 diluted FITC antibody (anti-mouse, Abcam, Cambridge UK) during 15 minutes washing and centrifugation steps, the pellet was resuspended in 150-300µl 0.5% BSA/PBS.

Cell counting assays

A first fluorescent cell sorting measurement for cell quality performance was conducted by Cyan analysis (Beckman Coulter, Brea CA). Subsequently, the cytokeratin-19 positive cells were sorted by a five-colour flow cytometry (FC500) according to a standardized protocol established during test settings (Beckman Coulter). Negative controls were routinely included. Cytokeratin-19 positive cells (cholangiocytes) were collected in 50-100µl 0.5% BSA/PBS and stored at -20°C.

Cell lysis and whole genome amplification

For 8 cases hepatic cyst DNA isolation and amplification of all samples were processed simultaneously. Cell lysation and DNA amplification was performed using whole-genome-amplification (WGA, Repli-G single cell kit, Qiagen, Hilden Germany).[17]

DNA Isolation and Sanger sequencing

Genomic DNA was extracted from blood leukocytes using the HP-PCR Template Preparation kit (Roche Applied Science, Penzberg Germany). PCR products from 8 subjects for all exons of the *PRKCSH, SEC63* and *LRP5* genes were analyzed by traditional Sanger sequencing on ABI310 or ABI3100 Genetic Analyzers (Applied Biosystems, Carlsbad, CA).

Genetic Analysis by Microarray and Whole-Exome Sequencing

Whole genome amplified DNA from hepatic cyst cells and genomic DNA from all 8 patients were analyzed using the CytoScan Genome-Wide Human SNP array (Affymetrix, Santa Clara CA). Capture of exomes from patient #1 was performed using Agilent's v4 (50Mb) exomes, and SOLiD sequencing (5500XL sequencer; Life Technologies). Sequence reads were mapped to the reference human genome (hg19) using LifeScope v2.1 (Life Technologies). Called variants were annotated with an in-house pipeline containing information from dbSNP134.[18, 19] We restricted our analysis to rare homozygous variants, encompassing regions of homozygosity that were identified in patient 1. Variant filtering was applied as previously reported.[19-22] In brief, we only selected non-synonymous variants affecting coding exons and canonical splice sites in chromosome 3, 9 and 13. Rare variants (frequency of <0.25% in both dbSNP134 and our in-house database containing >5,000 exomes) with high quality were analyzed.

Data Analysis

Per case all large regions of homozygosity of the autosomes (>3.0Mb) and large CNVs (>1.0Mb) were considered in the cyst-derived DNAs. Regions spanning the centromere were excluded because of the high degree of false positive findings. For each case a comparison of the array results derived from cyst cell DNA (somatic) and germline DNA (genomic) was performed to ensure the identification of somatic events. Genes in LOH >3.0Mb were prioritized by Mouse Genome Database (MGD) with the criteria phenotype (liver/ biliary phenotype, tumorgenesis or growth/ size phenotype, and (fetal) liver tissue expression (Genedistiller) for filtering and analyzing of possible candidate genes.[23] Unexpectedly, large germline events, i.e. CNVs >1.0Mb and regions of autozygosity (>5.0Mb) were also further addressed.

RESULTS

Patient Characteristics

From eight unrelated patients with a clinical diagnosis of PCLD cyst fluid was processed and hepatic cyst cholangiocytes were isolated (Table S1 and S2). All patients had severe symptoms and the mean age was 58 (range 44-83) years (Supplementary Text). One patient (patient #8) harbored a pathogenic mutation, *PRKCSH c.292+1G>C* (p.D98AfsX100). The remaining seven patients showed no causative mutation on the *PRKCSH*, *SEC63* or *LRP5* gene. Consanguinity was not reported in these affected individuals.

Performance of Cholangiocyte Sorting

In 89% of the aspiration procedures the cyst fluid was turbid or contaminated. The first hepatic cyst aspiration in patients frequently generates elucidated cyst fluid without macroscopic contaminants, or many blood or bile. This is the most favorable material for the work-up of cholangiocyte isolation. Hundreds to thousands cells were sorted.

Large Somatic LOH Identified Hepatic Cyst DNA

Patient #8 with germline *PRKCSH* mutation presented a large somatic LOH encompassing the *PRKCSH* conform with the second-hit hypothesis (Table 1). The only other obvious somatic hit identified >30Mb homozygous region of chromosome 16p in patient #7. Because our patients had no renal disease or extra-renal ADPKD symptoms, we prioritized genes associated with these somatic events according to tumorgenesis, growth/ size and liver/ biliary mouse phenotype, and (fetal) liver expression.

Large Germline Events

Our analysis also allowed the identification of large germline events. Remarkably, we identified a large CNV (2.9Mb) in patient #1 as well as several regions of homozygosity. This CNV in chromosome 9 has a low frequency in a normal, healthy cohort. Also in patient #7 we identified large regions of homozygosity in the blood derived DNA (Table 2).

Table 1. Identified mosaic 16p-isochromosome and LOH region in somatic DNA. Highly interesting genes are indicated in bold. *Selected genes by Genedistiller (criteria: MGD phenotype liver/biliary, growth/size or tumorgenesis, and (fetal) liver expression)[22]

Pt	Sample g.DNA	Sample cyst DNA	Chr.	Candidate genes*	Region cyst	Size (Mb)
7	PN01-1296	PN01-1128	16p	*LMF1, SOX8,* ***SSTR5****,* ***CACHA1H****, CLCN7, IGFALS, MSRB1,* ***TSC2****,* ***PKD1****, MLST8, E4F1, DNASE1L2, CCNF, PDPK1, CLCN6, TNFRSF12A, MEFV, CLUAP1, NLRC3, SLX4,* ***CREBBP****, VASN, DNAJA3, MGRN1, RBFOX1, PMM2, USP7, CLEC16A, SOCS1, ERCC4, MKL2, NTAN1, RRN3, MYH11, SMG1, COQ7,* ***UMOD****, SCNN1G, SCNN1B, GGA2, PALB2, PLK1, CACNG3, RBBP6, KDM8, IL4R, EIF3CL, CLN3, EIF3C, SH2B1, CD19, LAT, SPN, TAOK2, TBX6,* ***MAPK3****, MYLPF, ITGAL, CTF1, HSD3B7, BCKDK, KAT8, PRSS8, FUS, PYCARD, SLC5A2*	LOH cyst complete 16p	34.6
8	PN01-1107	PN01-1108	19p13.3-13.11	*PTPRS, SAFB, MLLT1, C3, INSR, MCOLN1, PNPLA6, RETN, CLEC4G, MAP2K7, ELAVL1, CCL25, ANGPTL4, PIN1, COL5A3, ANGPLT6, DNMT1, S1PR2, ICAM1, TYK2, KEAP1, CDKN2D, ILF3, DNM2, CARM1, SMARCA4, LDLR, RAB3D, EPOR,* ***PRKCSH****, ECSIT, MAN2B1, JUNB, DNASE2, KLF1, FARSA, CALR, GADD45GIP1, DAND5, NFIX, CACNA1A, CC2D1A, RLN3, IL27RA, PRKACA, PTGER1, GIPC1, NOTCH3, BRD4, PGLYRP2, RAB8A, KLF2, SIN3B, PLVAP*	LOH cyst	12.9

Table 2. Identified large CNV and LOH regions in genomic DNA. Highly interesting genes are indicated in bold.

Pt	Sample g.DNA	Sample cyst DNA	Chr.	Candidate genes*	Region cyst	Size (Mb)
1	PN01-1290	PN01-1124	3p22.1-21.1	***CTNNB1***, *ULK1, VIPR1, CYP8B1, ABHD5, CDCP1, CXCR6, CCR1, CCR2, CCR5, LTF, TMIE, PTH1R, SETD2, PTPN23, SCAP,* ***CDC25A****, TREX1, COL7A1, CELSR3, IP6K3, ARIH2, LAMB2, GPX1, DAG1, MST1, IP6K1, TRAIP, SEMA3F, GNAI2, HYAL1, HYAL2, TUSC2, RASSF1, CACNA2D2, RAD54L2, RPL29, ALAS1, BAP1, STAB1, PBRM1*	Homozygous region in genomic DNA	12.7
1	PN01-1290	PN01-1124	9q22.2-22.31	*SYK, NFIL3,* ***ROR2***	Large germline CNV	2.9
1	PN01-1290	PN01-1124	13q22.1-31.1	*TBC1D4, UCHL3, LMO7, MYCBP2,* ***EDNRB***	Homozygous region in genomic DNA	5.4
7	PN01-1296	PN01-1128	1p33-32.3	*TAL, STIL,****CDKN2C****, NRD1, RAB3B, ZCCHC11, GPX7, SCP2, MAGOH, LRP8, DIO1, HSPB11*	Homozygous region in genomic DNA	6.9

Whole-Exome Sequencing

Because microarrays of blood-derived DNA showed large homozygous regions in patient #1, whole-exome sequencing of this genomic DNA was performed. These homozygous regions may indicate a candidate region for a recessive PCLD gene. Whole-exome sequencing in patient #1 showed 96% with ≥20x coverage, and captured the genetic alterations in chromosome 3, 9 and 13. These large regions of homozygosity presented no unique homozygous variant in genomic DNA.

Loss of Heterozygosity Reveals Possible Candidate Genes

Our analysis indicated several possible candidate genes for hepatic cystogenesis based on protein expression, function and/ or pathway. These findings are explained in the discussion.

DISCUSSION

This study describes the processes to obtain DNA of hepatic cyst epithelium cells from cyst fluids in PCLD patients for further studies. Genome-wide analysis showed large somatic changes such as LOH in chromosome 19p and 16p in two cases. In addition, surprisingly large germline events were identified. Large regions of homozygosity may be used to map possible novel recessive candidate genes in sporadic cases. This study shows that genome-wide analysis in somatic and genomic DNA is an alternative tool that may assess mechanisms of cyst formation as well as identification of candidate genes with the first hit.

Second hits are postulated as the general molecular mechanism for hepatic and renal cyst formation in ADPKD.[23-25] Recently, this loss-of-function model is described to be identical for hepatic cystogenesis in PCLD affected individuals.[7, 8] All these previous studies used surgical tissue sections to analyze hepatic and renal cyst DNA. These invasive cyst fenestrations are rather infrequent because in each patient the clinical indication and individual advantages of this procedure is highly considered. In addition, there exists a high risk of surgical complications in contrast to the less invasive hepatic cyst aspirations. Therefore, we choose for hepatic cyst fluids to extract cyst-lining epithelium cells (cholangiocytes) for genome-wide analysis. Subsequently, we expected to indirectly assess novel PCLD candidate genes.

One PCLD patient (#8) harbored a pathogenic mutation in the *PRKCSH* gene (*c.292+1G>C*, p.D98AfsX100). Hepatic cyst DNA from patient #8 presented a large LOH of 12.9Mb encompassing the *PRKCSH* gene. Sanger sequencing of the somatic DNA identified that *PRKCSH c.292+1G>C* was present in a homozygous state. This finding is in line with previous reports of somatic loss in polycystic livers. In 76% of *PRKCSH* mutation carriers a somatic second hit is present in hepatic cysts. This mechanism was previously described in cases with *PRKCSH* cases by hepatic cyst tissue studies[8], but can serve as a proof-of-concept here. This study is furthermore the first presenting cases in which cyst fluid derived cells were used to show this. Only a small clear aliquot of cyst fluid is sufficient for the work-up. Next, we assessed the somatic and genomic DNA in seven PCLD patients without pathogenic variants in *PRKCSH*, *SEC63* and *LRP5*.

Genome-wide study of chromosome 16p in patient #7 demonstrated extremely diminished heterozygous calls indicating complete homozygosity of the short arm (Figure 1A). Interestingly, this mosaic loss of 16p reveals multiple genes associated with cystogenesis. It includes a known ADPKD gene, *PKD1*, but also genes directed to the pathophysiology. For example, the *SSTR5* gene encodes for the somatostatin receptor which is currently the target of non-invasive management by somatostatin analogues.

Another interesting finding is a smaller somatic LOH region (~3Mb) in patient #6. This included identification of the *CHD1* gene which encodes for E-cadherin. This protein functions as a calcium-dependent cell-cell adhesion molecule with pivotal roles in epithelial cell behavior, tissue formation, and suppression of cancer. Previous histology studies identified loss of the E-cadherin protein in hepatic cyst tissue sections derived from PCLD patients.[23]

Following genome-wide study of somatic events, we assessed large CNVs and homozygous regions in germline DNA. Firstly, patient #1 showed multiple homozygous regions of which the largest is 12.7Mb on chromosome 3p22.1-21.1 (Figure 2). This region showed selected genes which may be relevant for hepatic and/ or renal cystogenesis. For example, the *CTNNB1* gene encodes a central protein in the Wnt signaling and controls cell proliferation and differentiation. Previous studies reported that β-catenin interacts with polycystin-1 through the ubiquitin-dependent proteasome pathway.[26] Secondly, for the *CDC25A* gene is reported that protein overexpression leads to hepatic cystogenesis. Cell cycle progression and hyperproliferation can be targeted by Cdc25A blockers, for example vitamin K3 suppletion in rodent models.[25] In addition, the 5.4Mb homozygous region included detection of the *EDNRB* gene which is reported in rodent models as a major modifying factor for cystic disease progression in ADPKD.[27]

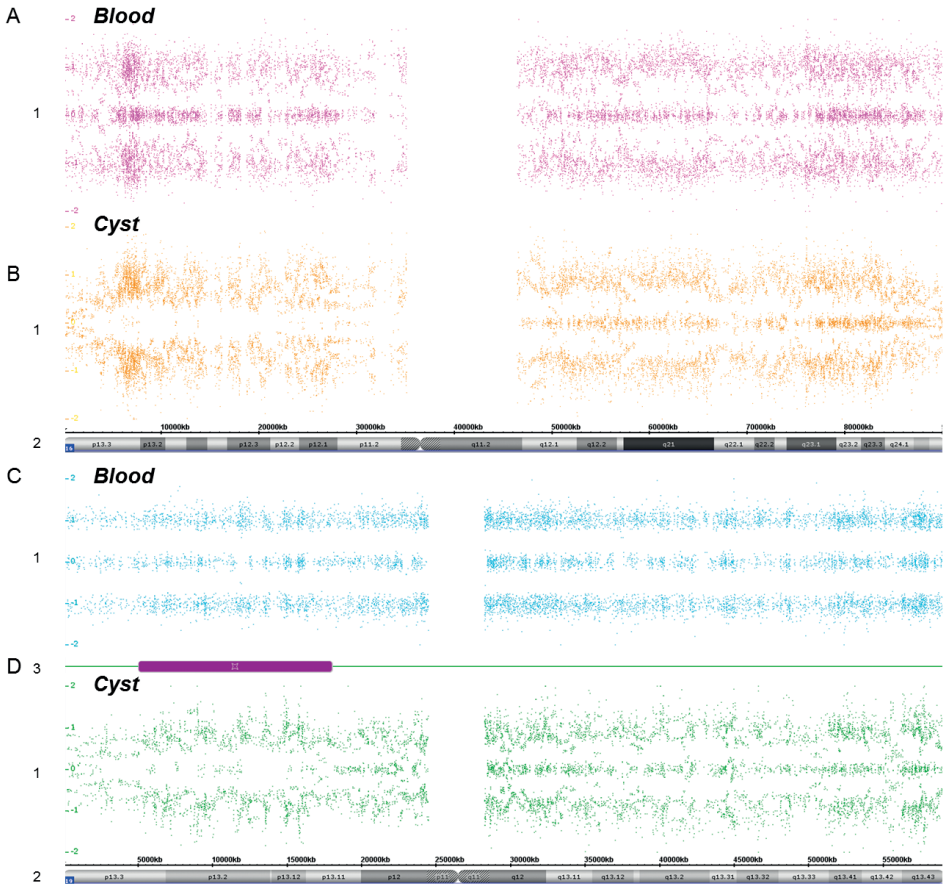

Figure 1. Microarray detection from hepatic cyst epithelium cells-derived DNA. (A) Microarray of chromosome 16 from germline DNA in patient #7. (B) Chromosome 16p with somatic LOH in patient #7. (C) Chromosome 19 from blood-derived DNA in patient #8. (D) Homozygous region in hepatic cyst DNA from patient #8 with a germline *PRKCSH* mutation. The numbers 1 to 3 chronologically indicate B-allele frequency, chromosomal position and LOH call.

Surprisingly, we also identified a very large CNV loss of chromosome 9 (2.9Mb) in patient #1. This deletion encompasses three genes associated with tumorgenesis, growth/ size and/ or liver phenotype, including *ROR2*. Deletions of this region and size are extremely rare, and make it a possible candidate region for a novel dominant PCLD gene. Although we have no direct proof that this deletion is causally linked to the PCLD phenotype, *ROR2* is a known protein in the Wnt signaling pathway and somatic loss has been described in tumorgenesis. A reduced expression of the tumor suppressor gene *ROR2* in hepatocellular carcinoma tissue sections was identified leading to impaired canonical Wnt signaling.[28]

A large homozygous region of 6.9Mb revealed multiple candidate genes relevant for cystogenesis in patient #7. *CDKN2C* is a tumor suppressor gene which contributes to medullary

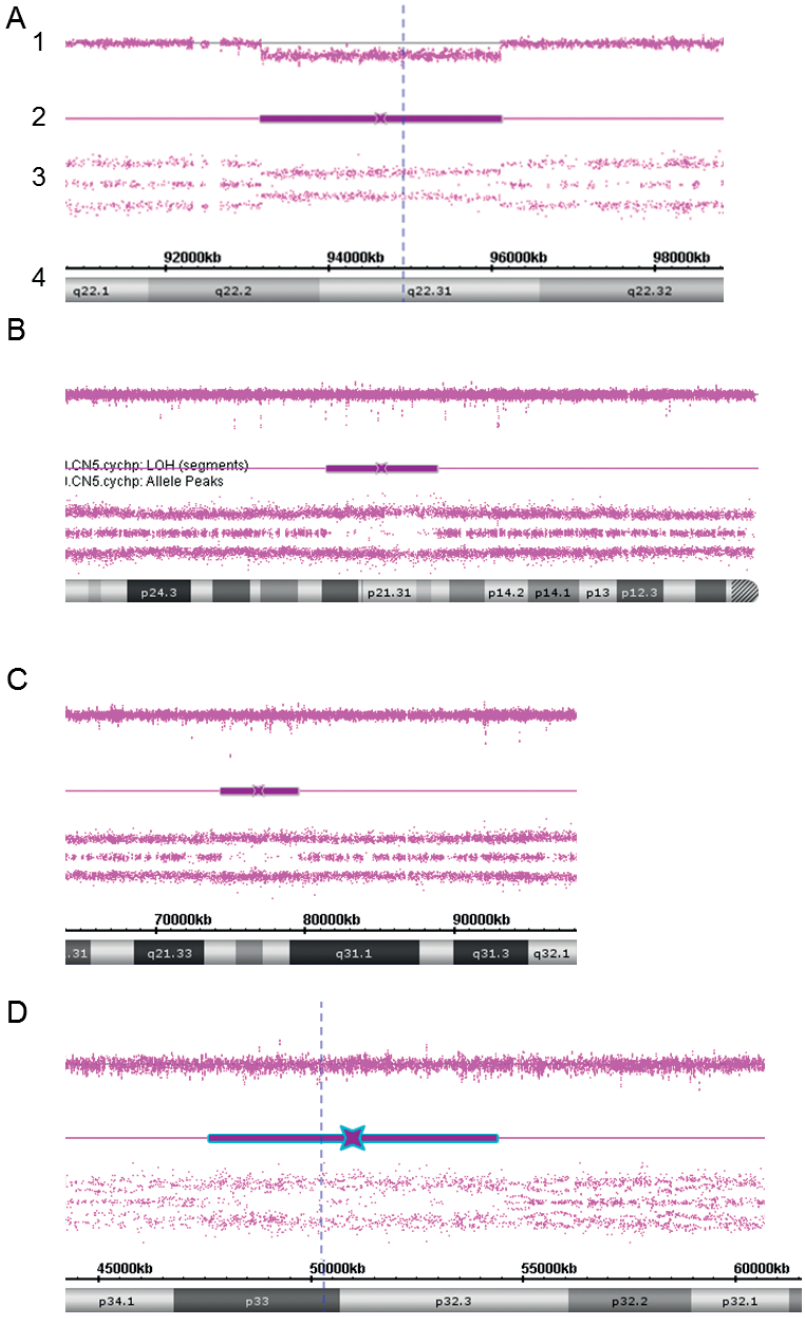

Figure 2. Microarray detection from blood-derived DNA. (A) A large CNV of 2.9Mb on chromosome 9 in patient #1. (B) Homozygous region of 12.7Mb on chromosome 3 in patient #1. (C) Homozygous region of 5.4Mb on chromosome 13 in patient #1. (D) Homozygous region of 6.9Mb on chromosome 1 in patient #7. The numbers 1 to 4 chronologically indicate the $\log_2$ intensity ratio, LOH calls, B-allele frequency and the chromosomal position.

thyroid carcinoma, Hodgkin lymphoma and feochromocytoma for example.[29] Somatic loss of *CDKN2C* suggests a direct role of selective growth advantages.

PCLD classically develops through a germline mutation in an autosomal dominant fashion. Although the hepatic cystogenesis is a developmental process, somatic DNA alterations may be early events in PCLD without onset of symptoms. Our findings present a high frequency of homozygosity in germline DNA. This is a remarkable finding in the normal human population. If patients indeed stem from non-consanguine parents, the genes in these homozygous regions may have a role as a recessive gene.

We explored the somatic and genomic DNA of 8 unrelated PCLD patients. Our findings indicate a role for multiple mechanisms of cyst formation and methods for gene discovery. We believe that the molecular mechanism of hepatic cystogenesis is a loss-of-function model. Dissection of relevant genes in PCLD is a major issue to understand the pathophysiology. The role of genes associated with tumorgenesis is unclear. A multistep process of cystogenesis is not excluded because at which stage the second, somatic event occurs is unknown. These somatic genetic aberrations may present early in life or later. Therefore, genomic alterations may explain the heterogeneity in PCLD patients.

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SUPPLEMENTARY TEXT

Clinical Characteristics of Polycystic Liver Disease Patients

Patient #1

Four years ago this male patient presented to us with acute abdominal pain. For already five years he had abdominal distension with mechanical symptoms, dyspnea d'effort and early satiety. At the age of 37-year-old he had an acute myocardial infarct. He administered a β-blocker to control hypertension. There was a positive family history of polycystic liver disease in his mother and a cousin. Abdominal ultrasonography showed a polycystic liver with several small cortical cysts in both kidneys. Theoretically, these clinical features correspond to the ADPKD criteria.

He underwent two times an aspiration sclerotherapy at the age of 57 and enrolled the ELATE trial in 2010.[30, 31] During this clinical trial he was treated with octreotide (40mg/ 28days) and everolimus (2.5mg/ day) for 12 months. Next, he developed symptoms of a large hepatic cyst. After completion of the ELATE trial, aspiration sclerotherapy was indicated. The collected cyst fluid was proceeded for this study. In addition, he was treated again with a somatostatin analogue, lanreotide 120mg/ 28 days during the 6-month RESOLVE trial.[32]

Molecular diagnostics of both PCLD genes, *PRKCSH* and *SEC63*, identified no pathogenic variants.

Patient #7

This 82-year-old female from Indonesian origin presented increased abdominal distension. Her hypertension is under control using a calcium antagonist combined with a selective type 1 angiotensine II-receptor-antagonist and hydrochlorothiazide. The family history for polycystic diseases is negative. Both parents died when they reached the age of 90. She had no history of clinical important diseases and delivered 12 children. Presence of polycystic liver disease in these individuals is unknown. Two daughters died because of colon cancer or cervix cancer.

CT scanning showed a polycystic liver with large hepatic cysts. One large cyst was 15.1 x 11.2 x 11.6cm and located ventral. Another large, dorsal cyst measured 15.4 x 11.3 x 12.0cm. In addition, a multi-septal cyst (size 12 x 13 x 9cm) in the right kidney was present.

This patient was referred for aspiration sclerotherapy. Drainage of two cysts was performed which resulted in 200ml lucid, achromatic cyst fluid and 800ml lucid, brown cyst fluid. The first aspirate was pursued for this study. No further treatment was indicated.

Molecular diagnostics of both PCLD genes, *PRKCSH* and *SEC63*, identified no pathogenic variants.

Patient #8

A 44-year-old female patient presented to us with episodes of acute abdominal pain and pyrosis. Clinical examination revealed hepatomegaly and palpable abdominal swelling. Ultrasonography showed multiple cyst spread throughout the liver. Mechanical symptoms increased and she was referred for aspiration sclerotherapy of the largest cyst (13.3 x 12.3cm). No further treatment was indicated.

Her family history was negative for polycystic liver disease, hepatic or renal disease. Since family members had no symptoms of hepatic or renal disease, abdominal screening for polycystic diseases was not performed. In addition, the children were younger than 18-years-old. Molecular diagnostics identified germline mutation *c.292+1G>C* in exon 4 of the *PRKCSH* gene.[2] This mutation is a known pathogenic variant. Segregation analysis was not performed in asymptomatic family members.

SUPPLEMENTARY FIGURES

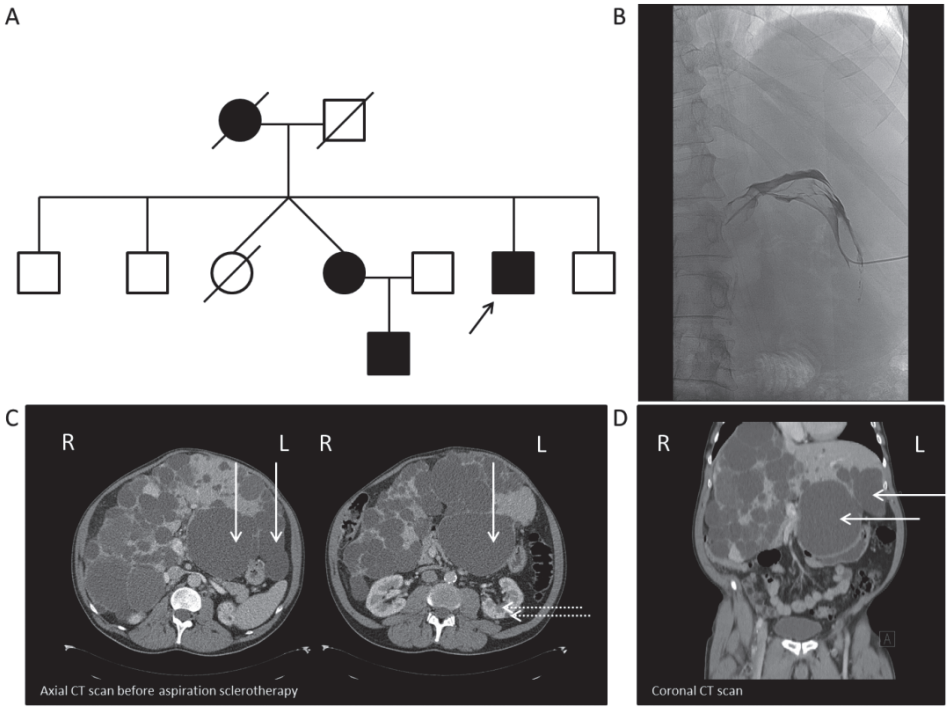

Figure S1. PCLD patient #1. (A) Pedigree of patient #1 indicated by an arrow. Squares indicate male sex, and circles indicate female sex. Solid symbols denote affected individuals, and open symbols are individuals without or unknown for PCLD. A slash indicates that the individual is deceased. (B) Positioning of the drain in the hepatic cyst during the aspiration sclerotherapy procedure. (C) Axial CT scan before aspiration sclerotherapy. (D) Coronal CT scan before aspiration sclerotherapy. The treated cysts are indicated by a white arrow.

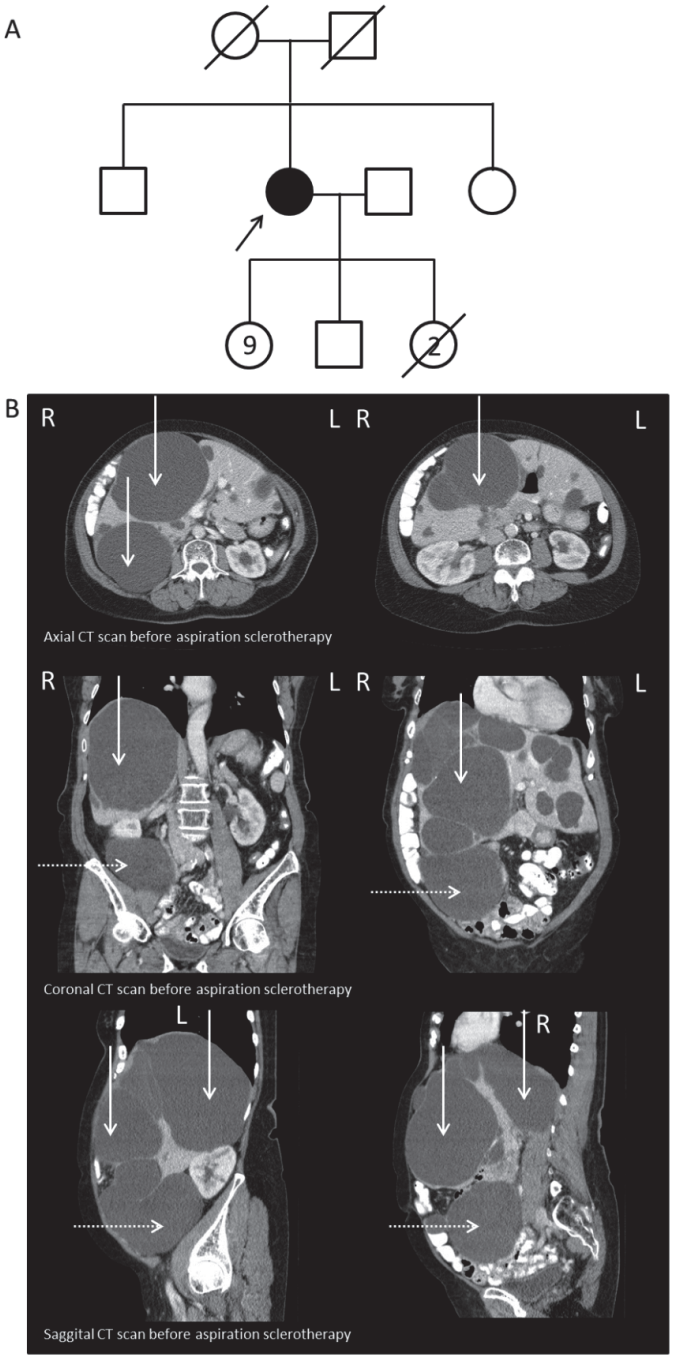

Figure S2. PCLD patient #7. (A) Pedigree of patient #7 indicated by an arrow. (B) Axial and coronal CT scanning of patient #7 indicating large hepatic cysts by white vertical arrows. Horizontal white arrows indicate a large renal cyst.

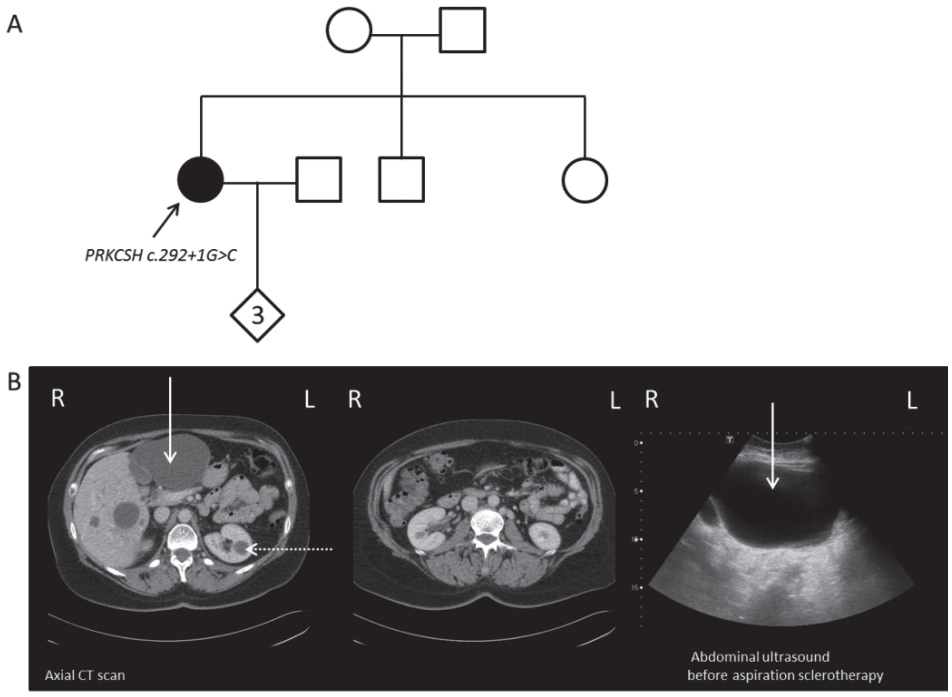

Figure S3. PCLD patient #8. (A) Pedigree of patient #8 indicated by an arrow. (B) Axial CT scan of patient #8. The third figure shows an abdominal ultrasound of the large hepatic cyst.

SUPPLEMENTARY TABLES

Table S1. Overview of collected hepatic cyst fluid samples.

Patient	Cyst fluid sample	Volume cyst fluid (ml)	Aspect of the cyst fluid	Sorted cells (n)
1.	242	1,020	Lucid, light-brownish	125,000
2.	247	290	Lucid, light-yellow	13,000
3.	250	70	Lucid, light-yellow	5,000
4.	253	1,300	Lucid, light-yellow	16,000
5.	264	2,400	Lucid, light-yellow	3,800
6.	266	1,100	Lucid, light-orange	25,000
7.	268	3,650	Lucid, achromatic	2,200
8.	274	490	Lucid, light-brown	8,500

Table S2. Patient characteristics per cyst fluid sample. *Age at aspiration sclerotherapy

Patient	Cyst fluid Sample no.	Sex (M/F)	Age (y)*	Liver and kidney phenotype	Germline mutation
1.	242	M	61	Severe polycystic liver; bilateral cortical renal cysts	WT
2.	247	F	58	Polycystic liver; cortical right renal cyst	WT
3.	250	F	60	Polycystic liver	WT
4.	253	F	50	Severe polycystic liver	WT
5.	264	F	59	Polycystic liver, large hepatic cyst	WT
6.	266	F	55	Polycystic liver, large hepatic cyst	WT
7.	268	F	83	Polycystic liver, large hepatic cysts, large renal cyst	WT
8.	274	F	44	Polycystic liver, large hepatic cysts	*PRKCSH c.292+1G>C*

CHAPTER 7

WHOLE-EXOME SEQUENCING REVEALS *LRP5* MUTATIONS AND CANONICAL WNT SIGNALING ASSOCIATED WITH HEPATIC CYSTOGENESIS

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ABSTRACT

Polycystic livers are seen in the rare inherited disorder isolated polycystic liver disease (PCLD) and are recognized as the most common extrarenal manifestation in autosomal dominant polycystic kidney disease (ADPKD). Hepatic cystogenesis is characterized by progressive proliferation of cholangiocytes, ultimately causing hepatomegaly. Genetically, polycystic liver disease is a heterogeneous disorder with incomplete penetrance and caused by mutations in *PRKCSH*, *SEC63*, *PKD1* or *PKD2*. Genome-wide SNP-typing and Sanger sequencing revealed no pathogenic variants in hitherto genes in an extended PCLD family. We performed whole-exome sequencing of DNA samples from two members. A heterozygous variant *c.3562C>T* located at a highly conserved amino acid position (p.R1188W) in the *low density lipoprotein receptor-related protein 5* (*LRP5*) gene segregated with the disease (logarithm of odds score 4.62), but was not observed in more than 1,000 unaffected individuals. Screening of *LRP5* in a PCLD cohort identified three additional mutations in three unrelated families with polycystic livers (p.V454M, p.R1529S, p.D1551N), again all undetected in controls. All variants were predicted to be damaging with profound structural effects on LRP5 protein domains. Liver cyst tissue and normal hepatic tissue samples from patients and controls showed abundant LRP5 expression by immunohistochemistry. Functional activity analyses indicated that mutant *LRP5* led to reduced wingless signal activation. In conclusion, we demonstrate that germline *LRP5* missense mutations are associated with hepatic cystogenesis. The findings presented in this study link the pathophysiology of PCLD to deregulation of the canonical wingless signaling pathway.

Keywords

Polycystic liver disease; Hepatomegaly; Whole-exome sequencing; Lipoprotein; Wingless signaling

INTRODUCTION

Polycystic liver diseases (PLD) consists of a group of inherited disorders characterized by abnormal proliferation and differentiation of bile duct epithelium.[1] Ductal plate malformation results in development of multiple fluid-filled cysts spread throughout the liver parenchyma. Progressive hepatic cystogenesis and cyst growth cause hepatomegaly and symptoms such as abdominal distension and pain, pyrosis, anorexia and dyspnea.

Congenital PLD are clinically heterogeneous and two major types can be distinguished. Autosomal dominant polycystic kidney disease (ADPKD) is a potentially lethal condition afflicting about 1:400 to 1:1,000 persons in the United States.[2] Patients may develop end-stage renal disease resulting from polycystic kidneys and 83% have the simultaneous presence of multiple hepatic cysts.[3] Formal diagnostic criteria for ADPKD include age, number of renal cysts for each kidney, and family history of renal disease.[4, 5] Isolated polycystic liver disease (PCLD) shares the phenotype of a polycystic liver, but is distinct from ADPKD because renal disease is absent.[6] Some patients may, however, have sporadic renal cysts. Diagnosis of PCLD is made by a family history consistent with autosomal dominant inheritance and presence of at least one (<40 years) or more than three (>40 years) hepatic cysts.[7]

The natural course of PCLD and ADPKD depends on a spectrum of factors that include age, sex, contraceptives, pregnancy and mutation(s).[8-10] Classical linkage analysis has identified four genes that underlie both inherited disorders. Mutations in *polycystic kidney disease 1* or *2* (*PKD1* or *PKD2*) genes (NM_601313 and NM_173910, respectively), which encode polycystin-1 and polycystin-2, are responsible for almost all ADPKD cases.[11-13] Both proteins are located in the cilium and are important in renal tubular cell morphogenesis and signaling functions, including the wingless (Wnt) signaling pathway (β-catenin pathway). Mutations in *protein kinase C substrate 80K-H* (*PRKCSH*) and *Saccharomyces cerevisiae homolog 63* (*SEC63*) are linked to PCLD (NM_002743.2 and NM_007214.4, respectively), and both gene products are located to the endoplasmic reticulum and are involved in processing and folding of glycosylated proteins.[14-16] In addition, the canonical Wnt signaling may be deregulated by the interaction partner nucleoredoxin of the Sec63 protein during oxidative stress.[17] In contrast to ADPKD, only 25% of PCLD cases can be explained by mutations in already known genes[18], indicating that mutations in several other as yet unidentified genes are involved in this disease. We hypothesized that another PCLD gene is involved in an extended PCLD family negative for the known disease genes and performed whole-exome sequencing. This unique approach has proven to be successful for the identification of the genetic cause of other Mendelian disorders.[19-21]

MATERIALS AND METHODS

Human Subjects

Ultrasound images of liver and kidneys were acquired using a 3.6-MHz general purpose clinical echo system (Acuson x150, SiemensAG) equipped with a curved linear array transducer. Blood samples were collected from all 40 subjects and DNA was extracted from blood leukocytes using the HP-PCR Template Preparation Kit (Roche Applied Science). Formalin-fixed paraffin-

embedded liver cyst tissue specimens were available from proband III/18 after laparoscopic cyst fenestration. DNA samples from 1,000 Dutch and 525 Moroccan healthy unrelated individuals were used as controls. All subjects provided written informed consent for all investigations. This study was approved by the Medical Ethics Committee of the Radboud university medical center, The Netherlands.

Sanger Sequencing

PCR products of proband III/18 for all exons of the *PRKCSH, SEC63, PKD1, PKD2* genes were analyzed by traditional Sanger sequencing on ABI310 or ABI3100 Genetic Analyzers (Applied Biosystems). Complete sequence analysis for *PKD1* variants was conducted by an experienced Center (Bioscientia). Exon and exon-intron boundaries of *LRP5* were determined using the Genome Bioinformatics Group of University of California, Santa Cruz (UCSC) Genome Browser, and unique primers were designed (Primer3). Heterozygous changes in *LRP5* were detected by high-resolution melting curves (RotorGene-Q; Qiagen) and validated by Sanger sequencing.

Genome Wide Copy Number Variation Analysis

DNA from whole blood was analyzed on the CytoScanHD which contains 2.6 million probes (Affymetrix). Hybridizations were performed according to the manufacturer's protocol. Genotype calls and copy number variation analysis were made using Affymetrix Chromosome Analysis Suite v1.2.0.225.

Whole-Exome Sequencing

Exome enrichment was performed using the SureSelect Human All Exon 50-Mb Kit (Agilent), covering ~23,000 genes. Emulsion PCR and bead preparation were made by using the EZbead system according to the manufacturer's instructions followed by SOLiD4 sequencing (Life Technologies). Reads were mapped to the hg19 reference genome using SOLiD Bioscope software v1.3 and annotated as described previously.[19, 20] All nonsynonymous variants shared in both affected individuals, absent or with very low frequency in dbSNP, were tested and validated in healthy and affected members of PCLD-1 family for cosegregation by high-resolution melting curve analysis and Sanger sequencing.

Linkage Analysis

We calculated the two-point logarithm of odds (LOD) score for linkage in the extended family using the SuperLink-v1.6 program in EasyLinkage-v5.08 software package. To determine the actual two-point LOD score for the *LRP5* mutation that was detected in this family, the mutation was considered to be a microsatellite marker in close proximity of *LRP5* (D11S4117). An autosomal dominant mode of inheritance was assumed with a penetrance of ~80%, and the disease allele frequency was estimated at 0.0001 (Supplementary Text).

Clinical Investigations

Because *LRP5* mutations are associated with bone density disorder, metabolic and ocular developmental diseases, we performed additional clinical investigations of members from the PCLD-1 family with a clear genotype-phenotype relation. An ophthalmologist (C.E.N.) performed an eye examination by a slit lamp to exclude retinal disorders such as familial exudative vitreoretinopathy (FEVR). At the same time, we analyzed metabolic and renal parameters in blood from these 22 individuals of the PCLD-1 family. We assessed bone density of the lumbar spine and left hip by dual energy X-ray absorptiometry (DXA scan; Hologic Discovery A, TROMP). Results are reported as T- and Z-scores which reflect the number of standard deviations by which a patient's value differs from the mean of a group of young or age-matched normal controls.

In Silico Analysis and Homology Modeling

We used three computational tools for the prediction of the functional effect of mutational variants (PolyPhen-2, Mutpred, and SIFT). LRP5 protein structure was created by using a LRP6 template as a start homology model and reconstruction by the YASARA&WHAT-IF Twinset. PDB files were available as templates for homology modeling of WD40 domains (β-propeller subdomains) to incorporate the identified extracellularly located *LRP5* mutations. Separate models were visualized for analysis of structural effects by YASARA.

Immunohistochemistry Studies

Formalin-fixed paraffin-embedded liver cyst tissue from proband III/18, four unrelated PCLD patients with a *PRKCSH c.1341-2A>G* mutation, and normal human liver tissue were available for immunohistochemistry studies. Staining intensity for the presence of LRP5 was compared between *LPR5* and *PRKCSH* mutants.

Expression Constructs

Total RNA was isolated from liver tissue using TRIzol Reagent (Invitrogen), and oligodT cDNA was obtained by RT Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences). Full-length WT *LRP5* was obtained using the Faststart High Fidelity PCR System (Roche). *LRP5* was cloned into the mammalian expression vector pcDNA3.1_V5_His TOPO-TA (Invitrogen) and checked by Sanger sequencing. An extracellularly *c.3562C>T* (p.R1188W) and intracellularly located *c.4651G>A* (p.D1551N) *LRP5* construct were generated by mutating the pcDNA.LRP5. WT vector using the Quick Change-II-XL Site-Directed Mutagenesis Kit (Agilent Technologies).

Luciferase Activity Assays

We used three cell lines to assess the functional effects of WT and mutant *LRP5*. For the activity assay $5.0x10^3$ Chinese hamster ovary (CHO) cells; $5.0x10^3$ human embryonal kidney 293 (HEK293) cells; $5.0x10^3$ Human cholangiocyte 69 (H69) cells per well were seeded in a 96-wells plate in triplicate. After 24 hours, cells were transiently transfected using X-tremeGeneHD (Roche) with 100ng *LRP5* construct ($LRP5_{WT}$; $LRP5_{R1188W}$; $LRP5_{D1551N}$), or empty vector and 100ng

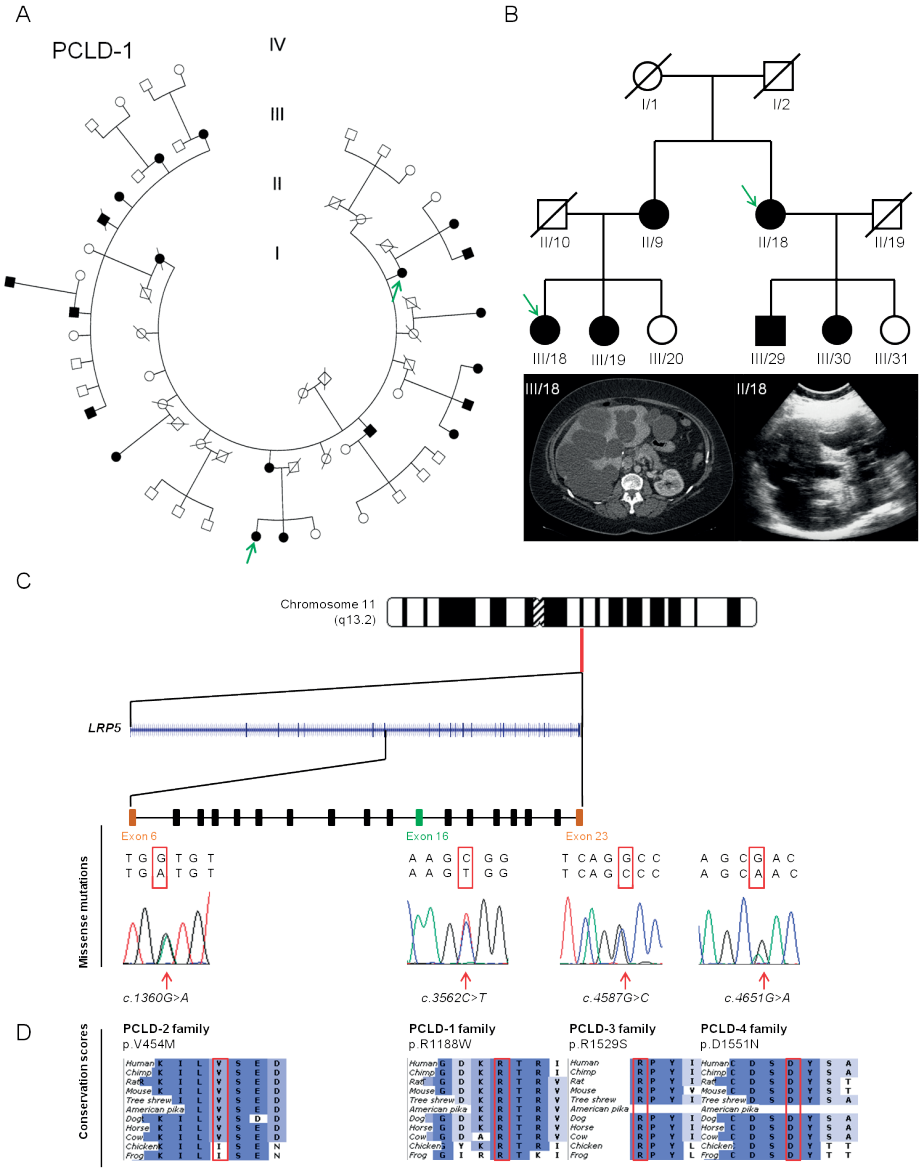

Figure 1. Identification of *LRP5* variants. *LRP5* variant p.R1188W in an extended Dutch PCLD-1 family (A) and three additional *LRP5* variants in three unrelated PCLD families were identified. Generations are denoted with Roman numerals, and individuals are numbered in a counterclockwise way. Squares indicate male sex, and circles indicate female sex. Solid symbols denote affected individuals, and open symbols are individuals without or unknown for PCLD. A slash indicates that the individual is deceased. (B) Simplified pedigree from PCLD-1 family with the clinical features by abdominal CT scanning and ultrasound of the liver of both probands (III/18 and II/18) in which whole-exome sequencing was performed (green arrow). (C) *LRP5* is located at chromosome 11q13.2, and the sequence electropherogram shows heterozygous germline mutations. (D) All *LRP5* missense mutations were located at highly evolutionary conserved amino acid regions with ortholog proteins from human to frog.

of TCF/LEF1 Reporter, or 100ng negative control according to manufacturer's instructions (Cignal TCF/LEF1 Reporter Assay Kit, Qiagen). Sixteen hours after transfection, medium was replaced by medium with or without 250ng/ml human Wnt3a (5036-WN, R&D Systems) to initiate Wnt signaling. Cells were cultured for another 24 hours, and luciferase activity was detected using the Dual-Glo Luciferase Assay System (Cat. No. E2920, Promega) in an InfiniteM200-Pro plate reader (Tecan). Firefly luciferase activity was normalized to Renilla luciferase activity for variations in transfection efficiencies. Values are reported as means ± standard deviation. These experiments were conducted in triplicate and performed three times. Western blotting was also performed to analyze possible differences in expression levels between the WT and mutant *LRP5*.

Quantitative PCR

We conducted transient transfections of HEK293 cells with *LRP5* constructs as described previously. The signaling was activated by addition of Wnt3a for 24 hours. Total RNA was extracted with TRIzol (Invitrogen). Template cDNA was obtained using the iScript cDNA Synthesis Kit (Biorad). Expression levels of Wnt target genes were assessed twice by quantitative PCR (qPCR) experiments (in triplicate) using the CFX96 real-time detection system (Biorad).

Immunofluorescence Imaging

A total of $4.0x10^4$ CHO cells per well were seeded on poly-L-lysine-coated Ø12mm cover glasses on a 24-well plate and transiently transfected with 100ng of $LRP5_{WT}$ or mutant *LRP5* construct. After 24 hours, medium was refreshed, and cells were cultured for another 24 hours followed by paraformaldehyde fixation and immunofluorescence staining.

Statistics

Groups were compared using descriptive statistics and compared by the Student T test. Both groups were compared by using the nonparametric Mann-Whitney U test. $P<0.05$ was considered statistically significant. All analyses were calculated by SPSS software v18.0.

RESULTS

Extended Polycystic Liver Disease Family

We assessed a 49-year-old female (III/18) by clinical history taking and physical and radiological examination. She presented to us with a severe symptomatic polycystic liver without renal cysts and complained of abdominal distension, pain, dyspnea and pyrosis. There was no history of renal disease, hypertension or intracranial aneurysms. She used contraconceptives for a total of 30 years and had 2 pregnancies. CT scanning revealed numerous cysts with diameters of 5-18 cm in liver segments I-III and VII. Despite aspiration sclerotherapy and laparoscopic deroofing of the liver cysts, her symptoms persisted, and she enrolled a clinical trial for long-acting octreotide treatment.[22] We expanded our clinical analysis and identified a 40-member Dutch family with three generations of early- and late-onset cystogenesis inherited in an autosomal dominant fashion (PCLD-1). Next, we studied 19 individuals with

cystogenesis of which 16 were affected with a polycystic liver according to the Reynolds criteria[5, 7] and 3 members affected with renal cysts that came from the 40-member Dutch family (Figure 1A). The proband (III/18) possessed no pathogenic variants affecting any of the known PCLD or ADPKD genes.

Whole-Exome Sequencing Identifies Pathogenic *LRP5* Variant

We performed exome sequencing on genomic DNA in 2 members (III/18 and II/18) from PCLD-1 family with an advanced polycystic liver phenotype (Figure 1B). Exome capture and sequencing were performed using the Agilent SureSelect target enrichment system with SOLiD4 sequencing. We identified 24,178 and 25,332 genetic variants per proband, respectively (Tables S1 and S2). Variants were annotated by a bioinformatics pipeline as described previously.[19, 20] Variant follow-up required the presence of at least five unique variant reads (different start sites), and the variant had to be present in at least 20-80% of all reads suggestive for heterozygous changes in a dominant model of disease. Next, we prioritized variants based on predicted amino acid consequences and overlap with common variation (presence in dbSNP v134 and/or an in-house database containing 1,300 analyzed exomes of predominantly European ancestry). We identified 11 unique nonsynonymous variants present in both affected relatives (Table S3). All 11 variants were tested for segregation in both probands and all other family members by Sanger sequencing. The only private nonsynonymous variant that cosegregated completely with the disease was a missense mutation (*c.3562C>T* with evolutionary conserved amino acid change p.R1188W) located on chromosome 11q13.2 in the *low density lipoprotein receptor-related protein 5* (*LRP5*) gene (NM_002335.2) (Figures 1C and 1D). We checked sequence coverage and excluded the presence of potential pathogenic variants in the 2 Mb surrounding *LRP5*. All 19 individuals with cystogenesis possessed *LRP5 c.3562C>T* (Table S4 and Figure S1). Analysis of the sequence data revealed linkage at the *LRP5* locus with the disease with a significant LOD score of 4.62 (Figure S2). This *LRP5* variant (*c.3562C>T*) was not detected in genome-wide sequence data from the 1,000 Genomes Project[23], 6,500 individuals from the National Heart, Lung, and Blood Institute Exome Sequencing Project[24], or exome data from 1,300 individuals of predominantly European ancestry sequenced in-house[19, 20], and excluded by Sanger sequencing in a control set of 1,000 Dutch DNAs of healthy, unrelated individuals. This *LRP5* mutation affected a highly conserved amino acid and was predicted to be damaging by PolyPhen-2, MutPred and sorting intolerant from tolerant (SIFT) models (Table 1).

LRP5 is a known disease gene causing severe skeletal bone or retinal disorders and is associated with metabolic disease (Figure S3). Therefore, we actively investigated the possible presence of subtle clinical signs in our family by slit-lamp eye examination. These investigations excluded FEVR in any of the family members (Supplementary Text). We assessed bone density of the lumbar spine and left hip in 13 patients and 9 unaffected relatives. The lumbar T-score was lower in *LRP5* mutation carriers, but within the normal range, and no member had a bone density disorder. Routine laboratory testing, including renal parameters, did not reveal differences between individuals with and without the *LRP5* mutation (Table 2). Therefore, other *LRP5*-related disorders were ruled out in this index family.

Table 1. Summary and *in silico* analysis of four *LRP5* variants in polycystic liver disease.

Family	Ethnicity	Genomic position chr.11 (hg19; GCRh37)	Mutation (*c.DNA*) (NM_002335.2)	Exon	Predicted effect on protein	PolyPhen2	MutPred	SIFT
PCLD-1	Caucasian (Dutch)	g.68193580	*c.3562C>T*	16	p.R1188W	Probably damaging (1.00)	0.552	Deleterious (0.00)
PCLD-2	Caucasian (Dutch)	g.68154128	*c.1360G>A*	6	p.V454M	Possibly damaging (0.872)	0.520	Deleterious (0.01)
PCLD-3	Moroccan	g.68216277	*c.4587G>C*	23	p.R1529S	Possibly damaging (0.610)	0.288	Deleterious (0.00)
PCLD-4	Caucasian (Dutch)	g.68216341	*c.4651G>A*	23	p.D1551N	Probably damaging (0.999)	0.235	Deleterious (0.00)

Additional LRP5 Variants in Polycystic Liver Disease

To confirm our results, we sequenced *LRP5* in a cohort of 150 unrelated PCLD probands without *PRKCSH*, *SEC63* or *PKD2* gene mutations. We identified two additional PCLD families (one Dutch and one Moroccan) and one Dutch PCLD singleton case with private *LRP5* missense variants. Mutations *c.1360G>A* (p.V454M) and *c.4587G>C* (p.R1529S) segregated with the disease in both families, and unaffected relatives of the singleton case (*c.4651G>A*; p.D1551N) did not carry the mutation (Figure S4).

Mutation *c.1360G>A* (p.V454M) was present in an 86-year-old polycystic liver patient. Her mutation-positive daughter had several bilateral renal cysts and small hepatic cysts on CT scanning. Her daughter died as a result of a metastasized liposarcoma at the age of 49. A 43-year-old Moroccan female had multiple dominant hepatic cysts without renal disease. Her 71-year-old mother had several hepatic cysts without renal cysts. Both carried the *c.4587G>C* (p.R1529S) mutation in the *LRP5* gene. A fourth mutation, *c.4651G>A* (p.D1551N), was present in a 65-year-old male with a polycystic liver and three renal cysts. There was no evidence for renal failure. The phenotype of his deceased parents was unknown, and both his healthy children were without hepatic or renal cysts, and did not harbor the *LRP5* mutation. None of the *LRP5* variants were detected in chromosomes from healthy, unrelated and ethnically matched controls (Dutch, n=1,000; Moroccan, n=525), nor present in the in-house or online exome sequencing datasets. In addition, all four *LRP5* missense variants affected highly conserved amino acids, and again were predicted to be damaging or deleterious by PolyPhen-2, MutPred and SIFT (Table 1).

LRP5 Expression in Liver Cyst Tissues

LRP5 has a wide tissue distribution, including liver and kidney, and expression has been reported in Kupffer cells, macrophages, stellate cells, cholangiocytes and hepatocytes.[25] In line with Northern blotting experiments[26], we found abundant LRP5 protein presence in normal liver tissues and relevant for PCLD. In the affected proband (III/18), we observed intense

Table 2. Baseline characteristics of PCLD-1 family.

	No hepatic cystogenesis and no *LRP5* mutation (n=9)	**Hepatic cystogenesis and *LRP5* mutation (n=13)**	**P-value**
Age – yr	55 ± 12	56 ± 13	NS
Female sex – no. (%)	6 (67)	9 (69)	NS
Bone density: Hip			
T-score Normal range -1.0-1.0 High-Normal range: 1.0-2.5	-0.19 ± 0.78	-0.08 ± 0.93	NS
Z-score Normal range -1.0-1.0 High-Normal range: 1.0-2.0	0.69 ± 0.68 *	0.95 ± 0.94	NS
Bone density: L1-L2-L3-L4			
T-score Normal range -1.0-1.0 High-Normal range: 1.0-2.5	1.18 ± 0.95 *	0.19 ± 0.88	0.042
Z-score Normal range -1.0-1.0 High-Normal range: 1.0-2.0	1.81 ± 0.89 *	1.18 ± 1.03	NS
Creatinin (µmol/l) Normal range 45-90	69 ± 14	73 ± 20	NS
Cholesterol (mmol/l) Normal 0.0-6.5	5.39 ± 0.71	5.44 ± 1.01	NS
GFR (MDRD) (ml/min/1.73m²) Normal >60	87 ± 13	85 ± 25	NS
BMI (kg/m²) Normal range 18.5-24.9 Overweight 25-29.9	25.7 ± 2.5	26.4 ± 5.2	NS
Triglycerides (mmol/l) Normal range 0.80-2.00	1.35 ± 0.71	1.80 ± 0.93	NS
HDL (mmol/l) Normal >1.10	1.34 ± 0.40	1.38 ± 0.32	NS
LDL (mmol/l) Normal range 3.5-4.5	3.44 ± 0.82	3.25 ± 0.78	NS
Non HDL (mmol/l) Normal range	4.06 ± 0.93	4.07 ± 0.93	NS
HbA1C (%) Normal range 4.0-6.0	5.5 ± 0.6	5.7 ± 0.8	NS
HbA1C (mmol/mol) Normal range 20-42	37 ± 6	39 ± 9	NS

NS, not significant. *There was one missing value because of degenerative abnormalities of the lumbar spine in an 85-year-old woman.

LRP5 staining of cyst-lining epithelium and bile ducts. The intensity of LRP5 expression was comparable in cyst tissue sections derived from a *PRKCSH* mutant and to bile duct epithelium (Figure 2A and 2B). This abundant LRP5 expression in cyst tissue indicates that there is no significant loss of structural LRP5 protein from relevant tissue in *LRP5* carriers.

For analysis of structural effects of the *LRP5* variants, we generated separated models for these domains (Figure 2E and 2F and Table S5).[27] The human LRP5 protein contains 1,615 amino acids and includes a long extracellular region, a single-span transmembrane, and a relatively short (208 amino acids) intracellular region. The extracellular region consists of four β-propeller domains with subsequent epidermal growth factor (EGF)-like domains. Near the transmembrane region are three LDL receptor class A repeats, whereas on the cytoplasmatic side, five PPPSP motifs are present. The β-propeller domains all consist of six segments of which most carry a characteristic YWTD motif. Arginine 1188 creates hydrogen bonds and ionic interactions in the core of the β-propeller structure, which is predicted to be lost by the tryptophan mutation (p.R1188W). Additionally, the larger tryptophan side-chain will cause steric clashes, which will most likely disturb the whole β-propeller domain. Valine 454 induces several hydrophobic interactions, but is also partly exposed to the solvent. Mutant methionine is predicted not to have major structural effects as both amino acids are hydrophobic and the methionine side-chain appears to fit at this position (p.V454M). Both p.R1529S and p.D1551N mutations are intracellularly located in between PPPSP motifs and an intracellular homology model is absent.

Reduced Activation of Canonical Wnt Signaling

To identify the underlying mechanism of mutated *LRP5* on the Wnt pathway, we conducted luciferase activity assays with $LRP5_{WT}$, one extracellularly ($LRP5_{R1188W}$) and one intracellularly located ($LRP5_{D1551N}$) mutant, and an empty expression vector as control. Immunofluorescence imaging in WT and both mutant constructs presented similar localization of LRP5 (Figure 2C and 2D). Western blots of the cell lysates showed comparable protein expression of all constructs (Figure 2G). Overexpression of the *LRP5* constructs in CHO cells increased basal and Wnt3a-induced luciferase activity compared with the empty vector ($P<0.0001$). In presence of Wnt3a, signal activation was significantly down-regulated by 30 and 45%, taken into account the basal activity in both *LRP5* mutants compared with the $LRP5_{WT}$. We also detected a significant decreased ($P<0.001$) activity of *LRP5* mutant p.D1551N without the presence of Wnt3a (Figure 2H). Luciferase activity assays were repeated in HEK293 and human liver-derived H69 cells, where comparable significant results were obtained (Figure S5).

Altered Expression Levels of Wnt Target Genes

Subsequently, we conducted qPCR experiments of transducers and transcription factors associated with the canonical Wnt signaling. Our analyses show that HEK293 cells transfected with mutant *LRP5* led to altered expression levels of target genes compared with $LRP5_{WT}$ (Figure S6). There was a significant increased gene expression of transducers adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), and transcription factor v-myc avian myelocytomatosis

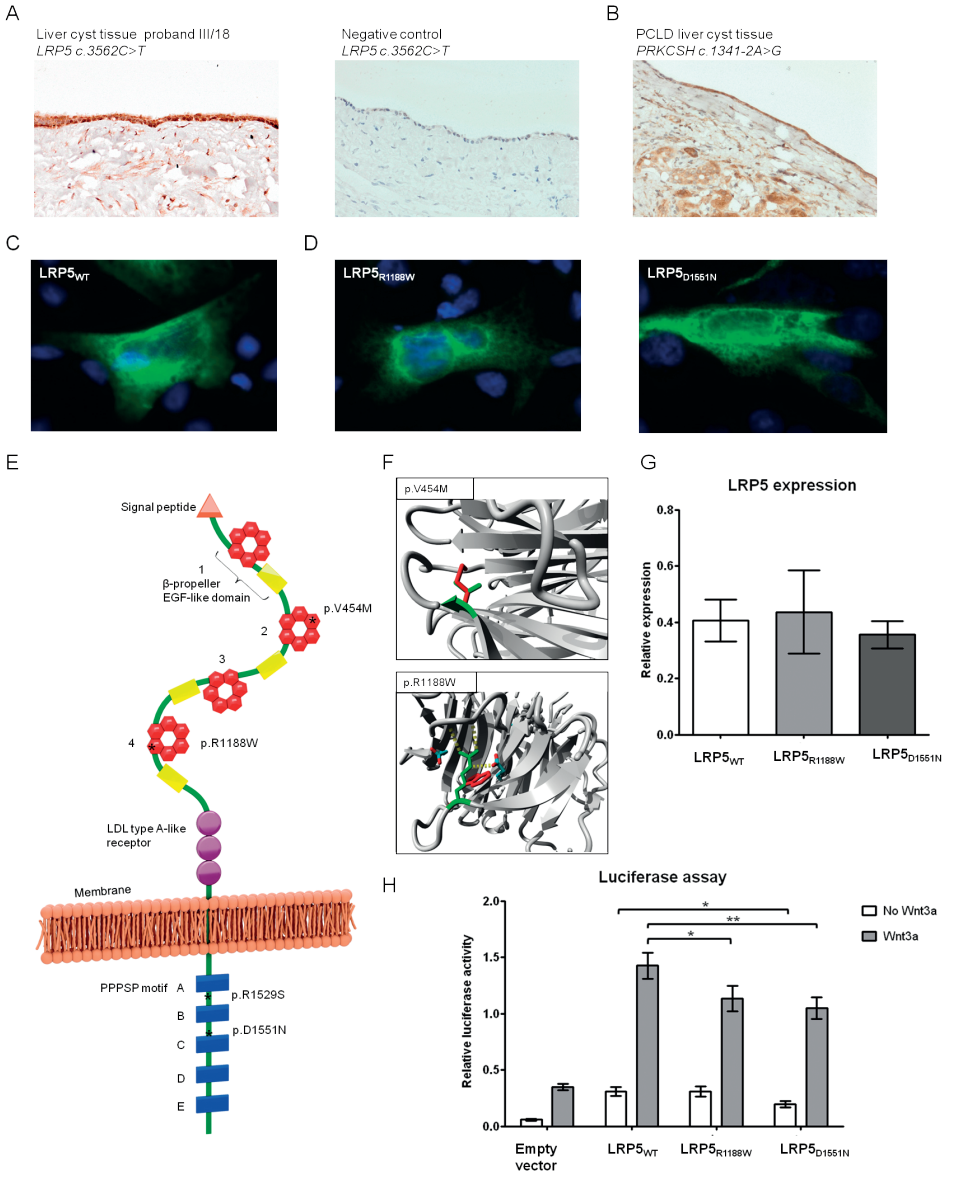

Figure 2. Functional and structural analyses of LRP5 variants in polycystic liver disease. (A) Immunohistochemistry of liver cyst tissue from proband III/18 of PCLD-1 family with *LRP5* mutation *c.3562C>T* (p.R1188W). The cyst lining cholangiocytes present positive staining for LRP5 compared with the negative control next. (B) PCLD patient with a *PRKCSH c.1341-2A>G* mutation shows similar staining of liver cyst tissue and expression of LRP5 compared with (A). (C) Localization of LRP5 protein was analyzed by immunofluorescence microscopy. CHO cells were transfected with constructs expressing $LRP5_{WT}$, (D) $LRP5_{R1188W}$, or $LRP5_{D1551N}$ and compared to negative controls. No differences in LRP5 localization between all constructs was detected. (E) Presentation of the human LRP5 protein and homology modeling of the β-propeller domains highlighting the amino acid changes to emphasize the impact on the configuration and surrounding protein domains. (F) Homology modeling of the LRP5 domains and detailed view of ▶

viral oncogene homolog (c-Myc) in $LRP5_{R1188W}$ or $LRP5_{D1551N}$, compared with $LRP5_{WT}$. Similarly, axis inhibitor-1 (AXIN-1), axis inhibitor-2 (AXIN-2), lymphoid enhancer-binding factor 1 (LEF1), SRY-box 9 (SOX9), fibroblast growth factor 18 (FGF18), and cyclin D1 (CCND1) were also up-regulated. In the presence of the extracellular regulator Wnt3a, expression levels of several Wnt signaling components changed (Figure S7). Decreased expression levels were found for GSK3β, AXIN-1, AXIN-2, LGR5, c-Myc, CCND1 and LEF1 compared with $LRP5_{WT}$ in both mutated *LRP5* constructs. These results are in line with the functional consequences of these *LRP5* mutations as shown with luciferase activity assays (Figures S8 and S9).

DISCUSSION

This study identifies *LRP5* as a novel gene associated with hepatic cystogenesis in patients clinically diagnosed with PCLD. The initial discovery was made in two affected relatives from an extended Dutch family by exome sequencing.[21] A private missense mutation *c.3562C>T* (p.R1188W) in the *LRP5* gene segregated with 18 affected relatives (>40 years), with a significant LOD score of 4.62. These findings are corroborated by the presence of three additional private missense *LRP5* variants in two PCLD families and one PCLD singleton. All unique variants identified in *LRP5* affected highly conserved amino acids and were predicted to be damaging or deleterious. The identification of *LRP5* as a causative gene follows that of *PRKCSH* (15%) and *SEC63* (6%) for isolated polycystic liver disease in a PCLD cohort.[18]

LRP5 is a single-span transmembrane protein that acts as a coreceptor with Frizzled protein family members for transducing signals by Wnt proteins. Wnt signaling directs a number of fundamental physiological mechanisms such as cell proliferation, cell polarity and cell fate determination during embryonic development.[28] Until now, *LRP5* variants were linked to a spectrum of Mendelian genetic diseases. *LRP5*-related disorders include autosomal dominant conditions with abnormal bone density, such as endosteal hyperostosis and osteosclerosis[29-32], but also in eye disorders such as recessive and dominant forms of FEVR.[29, 33, 34] Our findings expand the clinical spectrum of *LRP5*-associated phenotypes because there were no extrahepatic features in patients with *LRP5* mutations in our studies. Specifically metabolic disorders, diabetes mellitus, skeletal bone and retinal diseases were absent in the index family, and these were not reported for the other three families. We performed specific

▶ the amino acid changes shows extracellular *LRP5* mutation (*indicated) p.R1188W located at the sixth blade of the fourth β-propeller domain, and *LRP5* mutation p.V454M at the third blade of the second β-propeller domain. Both intracellular mutations p.R1529S and p.D1551N are located between PPPSP motifs A and B, and PPPSP motif B and C, respectively. (G) CHO cells were transiently transfected with empty, WT, or mutant *LRP5* vector. Whole cell lysate was analyzed by Western blotting using the V5 antibody and anti-β-actin. LRP5 protein expression levels (normalized to β-actin) are similar between $LRP5_{WT}$ and both mutant constructs $LRP5_{R1188W}$ and $LRP5_{D1551N}$. (H) Canonical Wnt signaling activity was analyzed by firefly luciferase activity and normalized to renilla luciferase activity with (in gray) or without addition of 250ng/ml Wnt3a. All *LRP5* constructs showed a significant increase in Wnt signaling activity compared with the empty vector ($P<0.0001$). Both *LRP5* mutants showed a decreased Wnt3a-induced signal activity (*$P<0.001$; **$P<0.0001$). Activity of $LRP5_{D1551N}$ without Wnt3a was significantly decreased compared to $LRP5_{WT}$.

clinical investigations in the extended family 1 and excluded FEVR in individuals with *LRP5* germline mutations by slit-lamp eye examination. Bone density measurements ruled out bone diseases in individuals with and without PCLD. Hepatic or renal cystogenesis has not been observed in association with FEVR or with bone diseases.

Why different *LRP5* mutations can result in such a wide spectrum of complex diseases that targets different tissues remains to be determined. LRP5 is detected by Northern blot analysis, immunohistochemistry and in situ hybridization studies in several tissues including the liver and kidney.[25, 26] Until now, *LRP5* mutations were linked to pathological retina or bone development. In the extended PCLD-1 family, we identified the *LRP5 c.3562C>T* mutation in 22 individuals, of which 19 had hepatic and/or renal cystogenesis. Two members were too young (<40 years) to develop hepatic cysts and one individual is an example of incomplete penetrance. Indeed, there was considerable clinical heterogeneity in affected members without complaints or patients with severe abdominal discomfort in the PCLD-1 family. This clinical heterogeneity has been described in families with *PRKCSH*- and *SEC63*-associated PCLD[14, 18], and penetrance of *PRKCSH*-associated PCLD is estimated at ~80%. Intrafamilial phenotypic variability suggests that modifier genes and/or environmental factors play a major role in PCLD disease expression. Similarly, the clinical expression of *LRP5*-associated bone and eye diseases is highly variable.[27]

There have been efforts to recapitulate the involvement of *LRP5* mutations in a number of bone and eye disorders in mice. A targeted knock-out $Lrp5^{tm1Jsak}/Lrp5^{tm1Jsak}$ mouse suffers from unspecified hepatobiliary abnormalities that might implicate phenotypic overlap with our observed human phenotypes.[35] Human and mouse LRP5 share a high degree of amino acid identity to coreceptor low density lipoprotein receptor-related protein 6 (LRP6) and have a similar domain structure.[36] Furthermore, there has been speculation about functional redundancy between both transmembrane coreceptors of the canonical Wnt signaling pathway.[37] The homozygous knock-out *Lrp6* mouse is embryonic lethal and possesses polycystic kidneys[37] compatible with the ADPKD phenotype. There is further experimental data to suggest that renal cystogenesis in ADPKD is linked to defective canonical Wnt signal transduction. Perturbations of polycystins cause inappropriate levels of β-catenin, and activation and inactivation of the Wnt signaling pathway are reported in different polycystic kidney disease mice models during embryonic or postnatal development.[38-42] Our findings provide a direct link between canonical Wnt signaling and polycystic liver disease as Wnt signaling is reduced in mutant *LRP5* compared with WT *LRP5*. A moderate reduction of signaling activity is in line with known *LRP5* missense mutations in retina and bone disorders.[43, 44] These observations support a role of an imbalanced canonical and noncanonical Wnt signal transduction in the pathogenesis of *LRP5*-associated polycystic diseases. We speculate that *LRP5*, along with other genes implicated in hepatic cystogenesis, *PKD1*, *PKD2*, *PRKCSH* and *SEC63*, is part of the functional genetic network. Experimental studies have observed that reduced dosage of these gene products, and in particular polycystin-1, is required to cause cyst formation.[45, 46]

The identification of *LRP5* associated with hepatic cystogenesis affords a better understanding of the pathophysiology of PCLD. Positivity for immunoreactive LRP5 in cyst

epithelium suggests that the protein is structurally intact. Functional assays in three different cell systems indicate that the mutation renders the protein to be less functional and that leads to inhibition of the canonical Wnt signaling.[41, 42]

In conclusion, PCLD is a genetically heterogeneous disorder that may be caused by *LRP5* gene mutations. Our results link hepatic cystogenesis to dysregulated canonical Wnt signaling and are in line with data that were generated from ADPKD models.[41, 42] Polycystins modulate Wnt signaling during organ development and contribute to hepatic and renal cystogenesis.[42, 47, 48] Our study provides evidence that mutations in *LRP5* are related to hepatic cyst development and fuels the hypothesis that canonical Wnt signal transduction is important in polycystic diseases. It is possible that disruption of other (downstream) members of the Wnt signaling pathway may be associated with initiation of hepatic and renal cyst formation.

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SUPPLEMENTARY TEXT

Investigations in PCLD-1 Family

Ultrasonography

Ultrasound images of liver and kidneys were acquired using a 3.6 MHz general purpose clinical echo system (Acuson x150, Siemens AG) equipped with a curved linear array transducer in all 39 members of proband III/18. Presence or absence of hepatic and/or renal cysts was investigated and noted carefully by at least two clinicians (W.R.C., M.C., J.P.H.D.). These ultrasound examinations were conducted in all 39 members within 2 weeks. During the project, five members (II/13; III/13; III/14; III/21; and III/26) were reevaluated by abdominal ultrasonography (Table S4). Member III/22 could not undergo a second evaluation due to chronic health issues. A complete clinical analysis of the liver and kidney phenotype in these individuals by CT or MRI scanning was not ethically approved for this project.

Ophthalmoscopy

Indirect ophthalmoscopy of the fundus was performed after full mydriasis of the pupil of both eyes by tropicamide 0.5% and phenylephrine 5% (wt/vol) eye drops. By performing slit-lamp biomicroscopy (Kowa SL90 slit-lamp and Volk S +70D lens), the posterior and peripheral retina was examined with special attention to the configuration of the retinal vasculature and signs of FEVR. The ophthalmologist (C.E.N.) was ignorant for our whole-exome sequencing results and blinded for this examination in 20 individuals: 11 affected (with *LRP5 c.3562C>T*) and 9 healthy relatives (without *LRP5 c.3562C>T*) in the extended polycystic liver disease PCLD-1 family. These indirect ophthalmoscopy examinations were performed in all 20 members within 1 day.

Laboratory Parameters

At the same time, we analyzed metabolic and renal parameters in blood from 22 individuals of this family. All presented values within normal ranges and showed no significant differences between individuals with and without the *LRP5* mutation (Table 2).

Bone Densitometry

We assessed bone density of the lumbar spine and left hip by a DXA scan (Hologic Discovery A, TROMP). Results are reported as T- and Z-scores, which reflect the number of standard deviations below the average for, respectively, a young adult at peak bone density and an average person of similar age. No relative was known for a bone diseases, nor was this detected by DXA scanning. The lumbar T-score was lower in *LRP5* mutation carriers, but no relative had a severe bone density disorder. The T- and Z-scores were within the normal-high range: 1.0-2.5 for T-scores and 1.0-2.0 for Z-scores. No significant differences were observed in hip T- and Z-scores and nonpathogenic high-normal values (T- and Z-scores within normal range, -1.0 to 1.0; Table 2).

SUPPLEMENTARY METHODS AND RESULTS

Luciferase Activity Assays

We performed the luciferase activity assays three times (and in triplicate) in human embryonal kidney cells (HEK293; ATCC CRL-1573). These results were in line with the findings with the CHO cell line for each *LRP5* construct. Both cell lines, HEK293 and CHO cells, are well-established study models with good transfection efficiencies and applicable to assess functional mechanisms in various disorders.[58] Here, we present the results of the luciferase activity assay in transiently transfected HEK293 cells (Figure S5).

The H69 cells are SV40-transformed normal human liver cholangiocytes originally derived from normal human liver and have been extensively characterized.[44, 59] Therefore, we also conducted these experiments in these cells, again three times (and in triplicate), and comparable results were obtained (Figure S5).

Quantitative PCR Experiments

To elucidate the mechanism of *LRP5* mutations in PCLD, we conducted transient transfections of HEK293 cells as previously described. We activated the signaling pathway by addition of Wnt3a for 24 hours, similar to former experiments. Expression levels of Wnt target genes *APC*, *AXIN-1*, *AXIN-2* and *GSK3*β, and *c-Myc*, *CCND1*, *LEF1* and other target genes were assessed by RNA isolation and subsequently by qPCR experiments (twice in triplicate). These genes are listed at http://www.stanford.edu/group/nusselab/cgi-bin/wnt/

Our analyses show that HEK293 cells transfected by mutant *LRP5* led to altered expression levels of target genes compared with WT *LRP5*. Transfected cells expressed LRP5 >5,000 times more. This high number indicates adequate transfection efficiency.

When expression levels for each construct were corrected, there was a significant increased gene expression of APC, GSK3β, c-Myc and LGR5 in mutant *LRP5*, $LRP5_{R1188W}$ and $LRP5_{D1551N}$, compared to the wild-type *LRP5* (Figure S6). Also, AXIN-1, AXIN-2, CCND1, LEF1, SOX9, Wnt3a and FGF18 showed increased expression, but this was not significant.

Secondly, gene expression levels were compared between basal and activated signaling activity by addition of Wnt3a. We corrected activated signaling for basal gene expression for a better understanding of the consequences of mutant *LRP5* on the expression of genes in the up-regulated pathway. Our results indicate unchanged gene expression for APC. Decreased expression levels were found for GSK3β, AXIN-1, AXIN-2, LGR5 and FGF18 compared to WT in both $LRP5_{R1188W}$ and $LRP5_{D1551N}$ (Figure S7A and 7B).

The same holds true for target genes at the nuclear end point (Figure S7C). CCND1, LEF1 and c-Myc expression levels decreased in mutated *LRP5*. Our results for gene expression levels demonstrate similar effects compared to the luciferase activity assays.

Disease Model

Wnt signaling has essential roles in normal tissue development and tumor growth. Wnt signal transduction depends on several proteins interfering with the amount of cadherin binding (at the plasma membrane) and cytoplasmic β-catenin. In absence of extracellular Wnt ligand, the

cytoplasmic pool of β-catenin is depleted by the β-catenin destruction complex (proteasome complex) consisting of AXIN, APC and GSK3β (Figure S8A). The GSK3β phosphorylates sites of the β-catenin and subsequent destruction by ubiquitination and proteolysis is initiated.

Wnt binding activates cytoplasmic Dishevelled (Dsh) to form a signalosome for LRP5 phosphorylation and proteasome destabilization. Subsequently, β-catenin accumulates in the cytoplasm and may be translocated to the nucleas. Binding of β-catenin to Tcf promotes the transcription and expression of Wnt responsive genes (Figure S8B).

We translated our results to this basic scheme of linear canonical Wnt signaling (Figure S9). We identified no activated signaling by luciferase activity (LEF1/Tcf) assays in mutant *LRP5*. This result resembles our findings from abovementioned additional experiments. Although the signal transduction is affected in mutant *LRP5*, we hypothesize that the proteasome complex remains active. Second, we hypothesize that a portion of β-catenin accumulates in the cytoplasm, and some β-catenin is destroyed (indicated by a cross with interrupted lines). In addition, it has been proven that β-catenin also binds to cadherins at the plasma membrane in PCLD.[60] Therefore, β-catenin does not enter the nucleus to bind Tcf for modulation of expression levels of genes associated with proliferation.

LEF1 is able to activate transcription independently from the presence of β-catenin. We found increased LEF1 expression in mutated *LRP5*, but lower levels compared with WT after addition of Wnt3a. There could be other factors influencing the equilibrium between the membrane and cytoplasmic β-catenin.[61] The exact mechanism needs to be explored, and the network of protein interactions in this pathway is major and complex.

In conclusion, in presence of Wnt3a, signal activation is down-regulated in $LRP5_{R1188W}$ and $LRP5_{D1551N}$.

SUPPLEMENTARY FIGURES

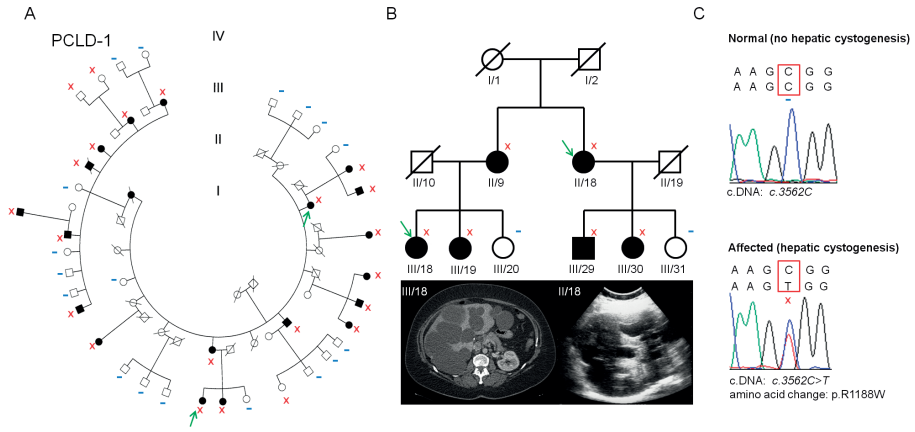


Figure S1. PCLD-1 family with genotype analysis. This additional figure illustrates the information from Table S4. (A) Pedigree of family PCLD-1 presents hepatic and/or renal cystogenesis phenotype (n=19, of which 16 with a polycystic liver) of the 40 members included for analysis (Figure 1) marked with genotype status (x in red; or - in blue). (B) Both affected relatives (II/18 and III/18) are indicated by an arrow (in green) and included for exome sequencing. (C) Figures S1A and S1C indicates which member possessed the heterozygous *LRP5 c.3562C>T* variant (x in red) and individuals with the normal variant (- in blue). Generations are denoted with Roman numerals, and individuals are numbered in a counterclockwise way. Squares indicate male sex and circles female sex. Solid symbols denote affected individuals, and open symbols are individuals without or unknown for PCLD. A slash indicates that the individual is deceased.

Figure S2. LOD score calculation in PCLD-1 family. The index family (PCLD-1) is a large pedigree ▶ segregating *LRP5 c.3562C>T* (p.R1188W). We calculated the LOD score in this family using the FastLink v2.51 program in the EasyLinkage v5.08 software package.[49] To determine the actual two-point LOD score for the *LRP5* mutation detected in PCLD-1 family, the mutation was considered to be a microsatellite marker in close proximity of *LRP5* (e.g., D11S4117; Figure S2). Both for the simulation and the LOD score calculation, an autosomal dominant mode of inheritance was assumed with a penetrance of 0.8 (80%), and the disease allele frequency was estimated at 0.0001. Individuals below the age of 40 (fouth generation; n=5) were not included in this analysis due to the age of onset of the phenotype. We analyzed the sequence data from 35 family members (n=18 members with cystogenesis; n=17 healthy members). Based on the number of individuals with *LRP5 c.3562C>T* (n=19), genome-wide SNP analysis combined with linkage analysis would likely have resulted in a genome-wide significant LOD score for a genomic region harboring the pathogenic mutation. Because we did not use SNP genotyping, in retrospect, we calculated the highest possible (maximum with θ=0) LOD score for this family (n=35), which was found to be 7.88. However, nonpenetrance is frequently observed in PCLD, which was supported by the fact that in this index PCLD-1 family, one healthy member and two individuals with bilateral renal (and splenic) cysts, without a hepatic cyst did carry the pathogenic mutation. Taking this into account, we assumed our mutation to be a microsatellite marker and included all family members above the age of 40 years that were genetically tested (n=35). Our analysis resulted in a two-point LOD score of 4.62 that, despite the presence of individuals without a hepatic cyst that carried the mutation (n=3), is considered to be genome-wide significant.

Project:	WYBRICH CNOSSEN	Inheritance:	Dominant	Marker	CHR	cM	LOD	Theta
Family name:	TOTALS	Common allele:	99.90 %	1.D11S4117	11	58.00	4.6181	0.0000
Used map:	LDB v2 (sex-averaged)	Disease allele:	0.10 %					
Marker positions:	1 ok / 0 ? / 0 outside	LC1 PCOPY rate:	0.00 %					
Allele frequencies:	All individuals from marker file	LC1 PENET wt/wt:	80.00 %					
CALC interval:	Entire chromosome	LC1 PENET mt/mt:	80.00 %					

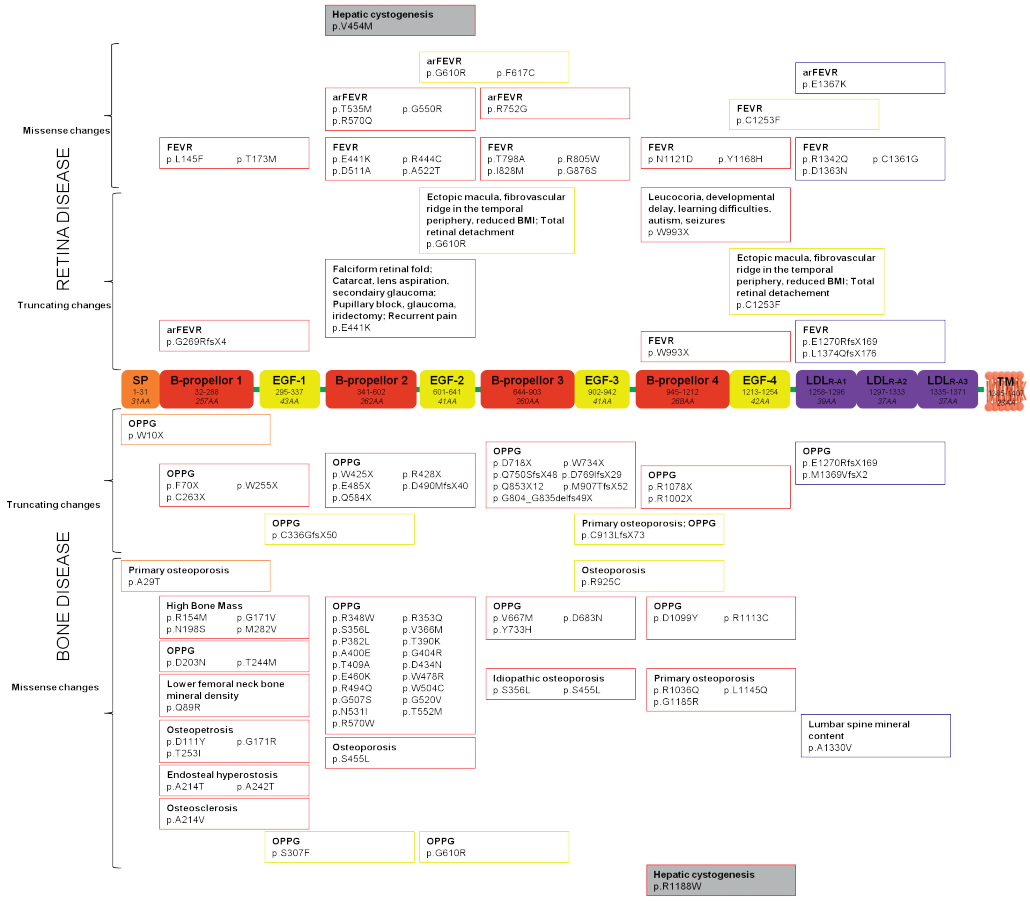
Figure S3. Extracellular and intracellular *LRP5* mutations. This figure summarizes the location and number of all nonsense and missense mutations in the highly conserved *LRP5* gene resulting in a truncated or an amino acid change of the LRP5 protein. There are 88/117 (75%) nonsense and missense mutations identified in the *LRP5* gene causing multiple complex diseases: autosomal dominant and autosomal recessive retina or bone diseases. Hepatic or renal cystogenesis has not been observed in association with FEVR or with bone diseases. ▶

A considerable genetic locus heterogeneity in *LRP5* is present. Mutations in the *LRP5* gene are spread throughout the extracellular (A) and intracellular LRP5 protein domains (B). One exception are missense mutations in high bone mass disease patients, which are only located to the first β-propeller domain in LRP5. Remarkably, more mutations at the extracellular LRP5 domains were identified. This overview indicates that the relationship between a retina or bone phenotype for specific domains or protein change is not obvious (unclear), which could explain the distribution of the identified *LRP5* mutations in polycystic liver patients.

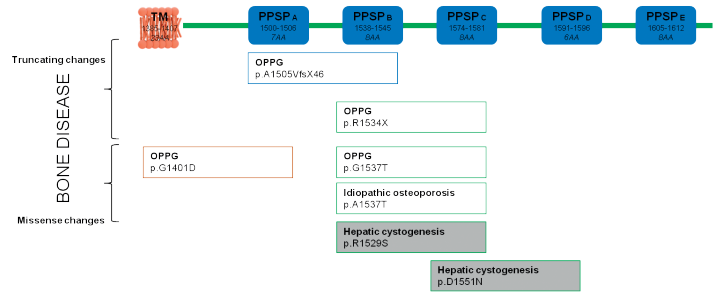
In conclusion, we identified four missense mutations in three unrelated families and one singleton patient with severe hepatic cystogenesis. All *LRP5* mutations were predicted to be damaging or deleterious with profound structural effects on the LRP5 protein, especially the extracellular domains. We have not detected these variants in either DNAs from healthy unrelated individuals from similar ancestry or assessed in the in-house data (1,300 individuals) and online sequence data (6,500 individuals from the ESP cohort; 1,000 Genomes Project).[23, 24] We performed additional clinical and functional investigations to underline our findings.

Protein domain information was derived from MRS database; v6 (latest version); http://mrs.cmbi.ru.nl/m6/entry?db=sprot&id=lrp5_human&rq=lrp5_human;[52] *LRP5* mutations were derived from a recent review by *Nikopoulos K. et al.*[27] and the Human Gene Mutation Database (www.biobase-international.com/hgmd).[53] Small indels (n=2), deletions (n=13), insertions (n=4) and splice site (n=8) mutations were not included in this figure.

A. EXTRACELLULAR LRP5



B. INTRACELLULAR LRP5



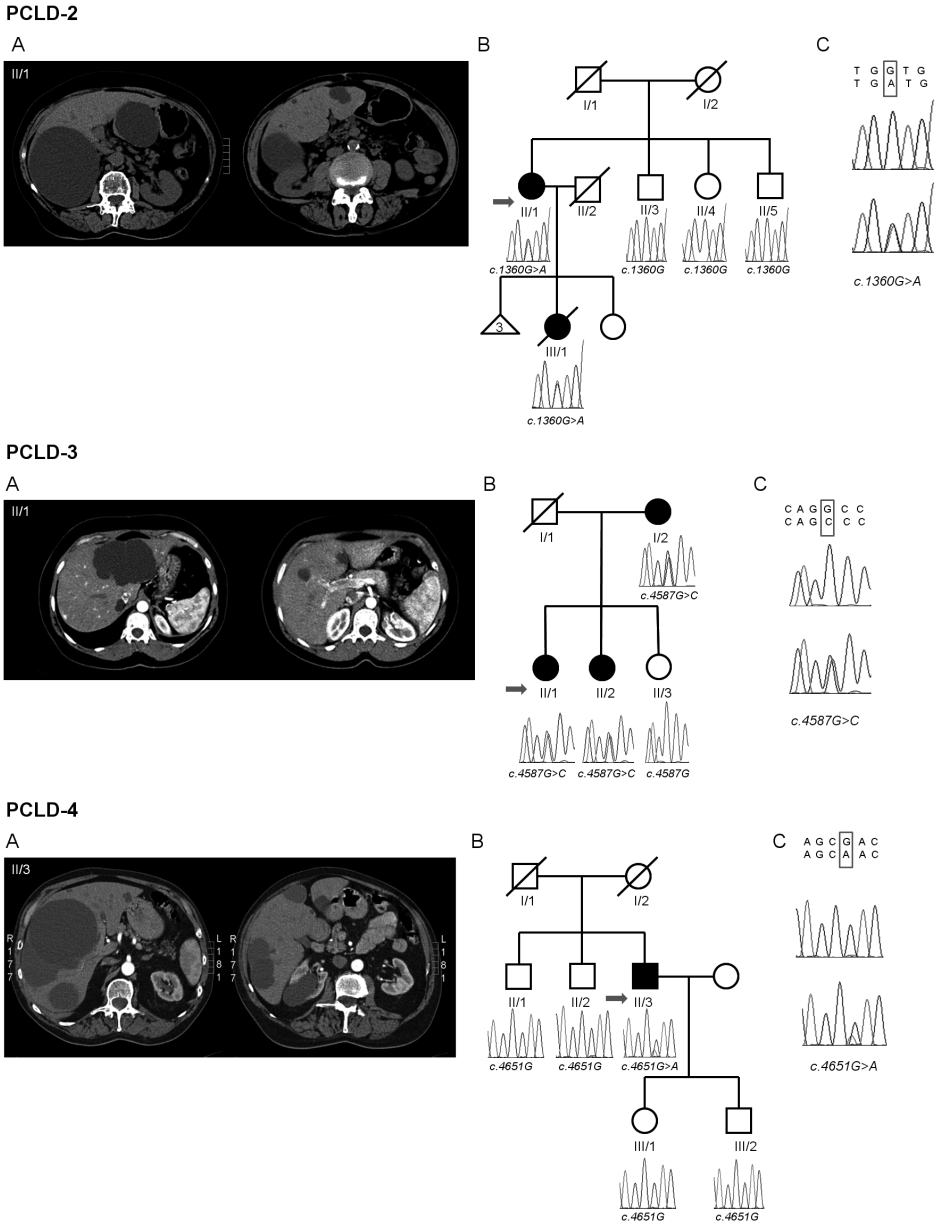


Figure S4. CT scanning of the proband and pedigree of three additional PCLD families with a *LRP5* missense mutation. The *LRP5* mutation cosegregated with the disease phenotype in PCLD families 1, 2 and 3. All missense mutations were predicted to be pathogenic. For each family, (A) CT scanning of the proband (blue arrow in B) and (B) the pedigrees of family PCLD-2, PCLD-3 and PCLD-4. (C) Positions of the mutations are denoted according to the hg19 human reference genome (GCRh37).[50, 51]
PCLD-2 family: *LRP5* g.68154128, c.*1360G>A*; p.V454M.
PCLD-3 family: *LRP5* g.68216277; c.*4587G>C*; p.R1529S.
PCLD-4 family: *LRP5* g.68216341; c.*4651G>A*; p.D1551N.

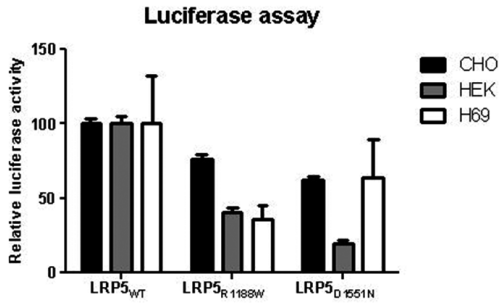


Figure S5. Luciferase activity assays. Luciferase activity assays for $LRP5_{WT}$ and both mutant constructs $LRP5_{R1188W}$ and $LRP5_{D1551N}$ performed in CHO, HEK293 and H69 cells. Firefly luciferase activity was normalized to renilla luciferase activity by calculating the ratio. Wnt3a-induced signal activity was reduced in all cell systems compared to the $LRP5_{WT}$ ($P<0.001$).

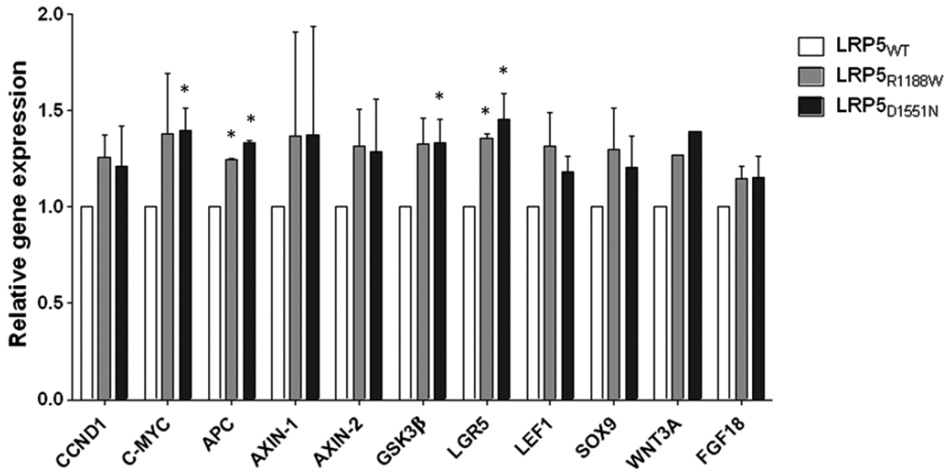


Figure S6. qPCR experiments. Basal gene expression levels of genes involved in the canonical Wnt signaling pathway (for example). No Wnt3a was added to activate signaling. We corrected results for $LRP5_{WT}$ levels. *Significant increased expression levels. The y-axis presents the relative gene expression level.

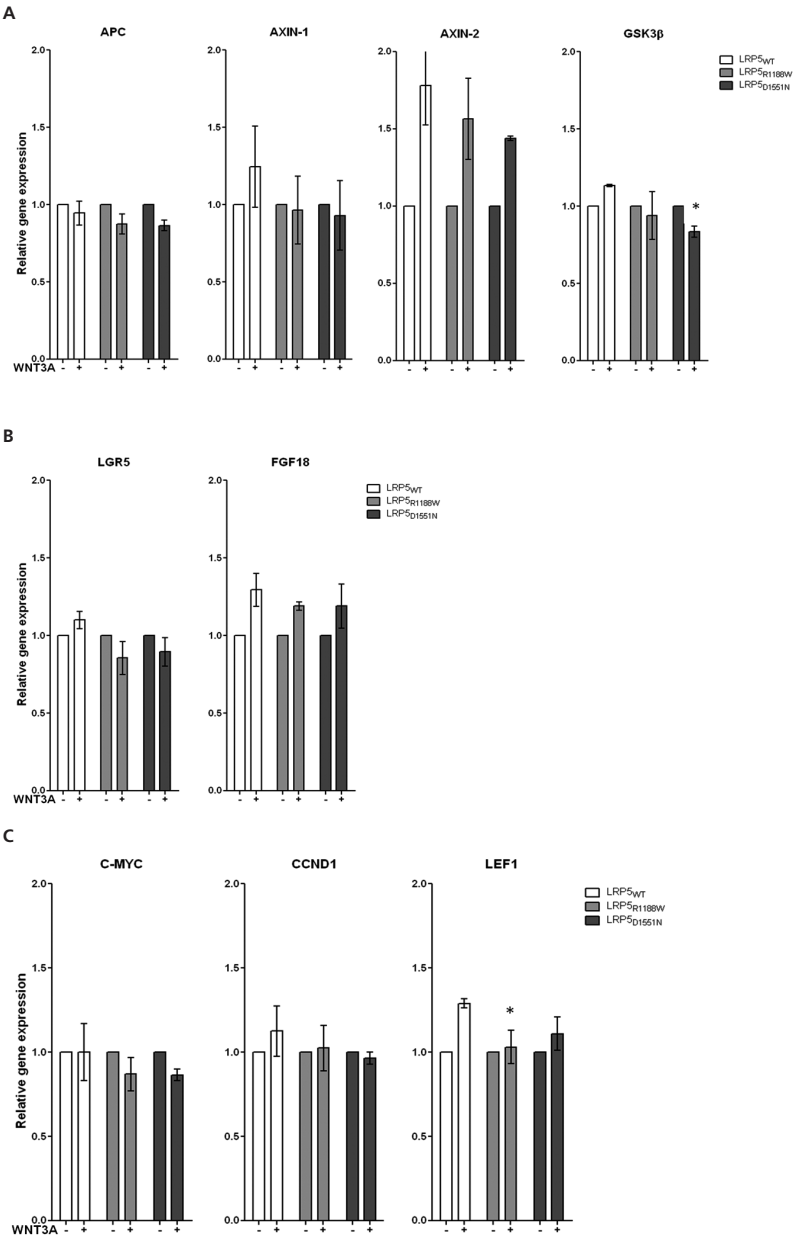


Figure S7. qPCR experiments. (A) Gene expression levels of APC, AXIN-1, AXIN-2, and GSK3β involved in β-catenin degradation. (B) Expression levels of other target genes of the canonical Wnt signaling. (C) Expression levels of target genes at the nuclear end point of the canonical Wnt signaling pathway. In all panels (A, B, C), the first bar presents the unstimulated situation (basal gene expression levels). Expression levels of the second bar are the result of activation of the Wnt signaling by extracellular ligand Wnt3a. Activated gene expression levels are corrected for basal gene expression of the respective $LRP5_{WT}$, $LRP5_{R1188W}$, and $LRP5_{D1551N}$ constructs. *Significant decreased expression levels. The y-axis presents the relative gene expression level.

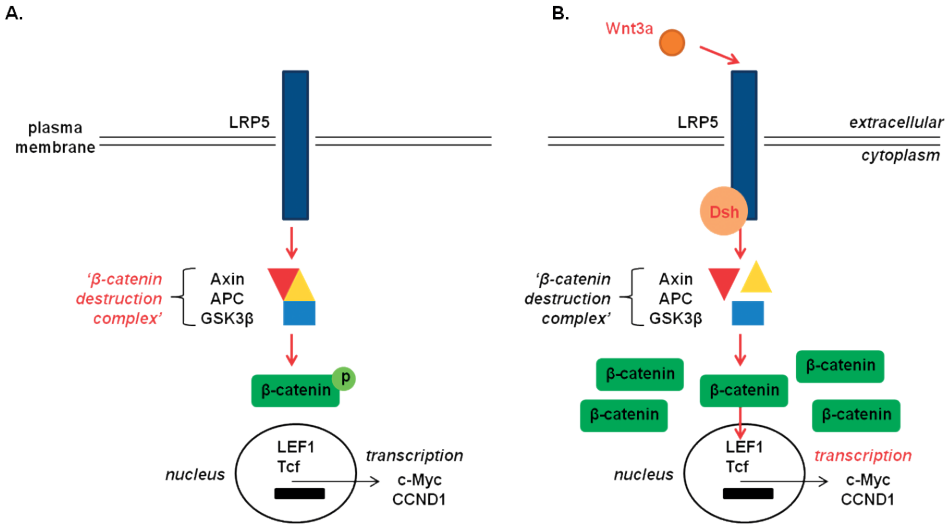


Figure S8. Canonical Wnt signaling under normal conditions. Canonical Wnt signaling without extracellular regulator (A) and activated with Wnt3a (B) under normal conditions. In red, the effects and signaling direction are indicated.

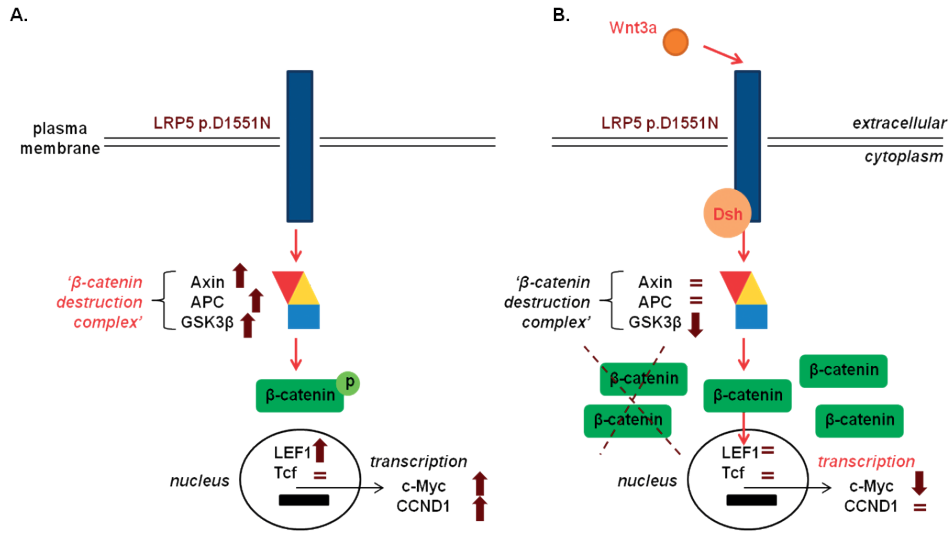


Figure S9. Disease model: proposed canonical Wnt signaling with LRP5$_{D1551N}$. Canonical Wnt signaling without extracellular regulator (A) and activated with Wnt3a (B) in PCLD (*LRP5* mutation p.D1551N). Brown arrows indicate the altered expression levels of the involved target genes from our experiments.

SUPPLEMENTARY TABLES

Table S1. Summary of whole-exome sequencing performance in two probands of PCLD-1 family.

	III/18	II/18
Total mapped bases in regions	3.9G (79.0%)	2.8G (76.8%)
Total mapped bases near regions	0.41G (8.3%)	0.30G (8.0%)
Total mapped bases outside regions	0.62G (12.7%)	0.56G (15.2%)
Total reads	126M	108M
Total mapped reads	103M (81.6%)	80M (74.13%)
0 mismatches	58M	36M
Uniquely placed reads	85M	64M
Median target coverage	58.07x	41.70x
% Targets covered ≥10x	83.9%	83.4%
% Targets covered ≥20x	76.5%	74.4%

Table S2. Prioritization of private variants.

	III/18	II/18
Number of called private variants*	24,178	25,332
Exonic and canonical splice sites	11,705	12,083
Non-synonymous and splice sites	5,743	6,004
Novel dbSNP v134[56]	334	373
Absent in in-house database**	72	11
Shared variants***	11	

*****Private variant: A unique nucleotide change (on DNA level) derived from an individual in which exome sequencing was performed (in this study). These private variants may be benign or pathogenic depending on many factors (for example size/ location/ effect on amino acid or protein). Interesting candidate genes for unexplained disorders in patients may be unknown private variants not listed as a known SNP (polymorphism) or have a very low frequency in a population. **In-house database: 1,300 whole-exome sequence data from individuals of predominantly European ancestry. *******Shared variant: A private variant that is present in two affected individuals (in this study: affected members III/18 and II/18).

Table S3. Eleven private variants shared in both affected probands from PCLD-1 family.

Chr.	Position (hg19)	Reference	Variant	Gene name	Amino acid change	PhyloP	Variant in members with PCLD*	Variant in members with no PCLD
chr1	g.247769018	C	A	OR2G3	p.T44N	0.17	6/16	4/16
chr11	g.65633353	G	T	MUS81	p.G526V	5.181	9/16	15/16
chr11	g.68193580	C	T	LRP5	p.R1188W	0.016	16/16	0/16
chr11	g.99932087	G	A	CNTN5	p.R375H	3.051	8/16	3/16
chr12	g.13102792	T	C	GPRC5D	p.Y176C	5.247	6/16	7/16
chr15	g.31341712	C	A	TRPM1	p.V458L	5.479	8/16	7/16
chr15	g.43712642	A	T	TP53BP1	p.R1509E	0.791	8/16	4/16
chr16	g.71674378	T	A	MARVELD3	p.S227W	-0.534	11/16	8/16
chr6	g.129581891	A	G	LAMA2	p.Y711C	0.302	8/16	9/16
chrX	g.105190439	G	A	NRK	p.V1446M	2.433	7/16	4/16
chrX	g.149840060	C	T	MTM1	p.H602Y	4.244	6/16	2/16

*Eight members excluded during high-resolution melting curve analysis and direct Sanger sequencing (<40 year old and/or renal cystogenesis/no PCLD): II/13, III/22, III/26, IV/1, IV/2, IV/4, and IV/5.

Table S4. Clinical presentation of PCLD families included for genotype analysis.

Inclusion (n=40) PCLD-1	Sex	Age (y)	Liver phenotype (n=16)	Kidney phenotype (n=12)	Diagnosis (PCLD n=16; renal cyst(s) n=3)	*LRP5* Mutation (n=22)
>40 years						
I/1	F	42†	n.a.	n.a.	n.a.	n.a.
I/2	M	70†	n.a.	n.a.	n.a.	n.a.
II/1 [$]	F	81†	Y	Y	PCLD	n.a.
II/3	F	47†	n.a.	n.a.	n.a.	n.a.
II/4	F	85	N	N	No PCLD	Normal
II/5	F	82†	n.a.	n.a.	n.a.	n.a.
II/7	F	72†	n.a.	n.a.	n.a.	n.a.
II/9	F	80	Y	N	PCLD	p.R1188W
II/11	F	79†	n.a.	n.a.	n.a.	n.a.
II/13 **	M	78	N	Y	Renal cysts	p.R1188W
II/15	M	56†	n.a.	n.a.	n.a.	n.a.
II/16	F	76†	n.a.	n.a.	n.a.	n.a.
II/18 [2E]	F	72	Y	Y	PCLD	p.R1188W
II/20	F	57†	n.a.	n.a.	n.a.	n.a.
III/1	F	58	Y	Y	PCLD	p.R1188W
III/3	F	56	Y	Y	PCLD	p.R1188W
III/5	F	55	Y	Y	PCLD	p.R1188W
III/6	M	54†	Y	N	PCLD	p.R1188W
III/7	F	52	N	N	No PCLD	Normal
III/9 [#]	M	51	Y	Y	PCLD/ADPKD	p.R1188W
III/10	F	50	N	N	No PCLD	Normal
III/11	M	47	N	N	No PCLD	Normal
III/12	M	46	N	N	No PCLD	Normal
III/13	M	42	Y	Y	PCLD	p.R1188W
III/14	F	44	Y	N	PCLD	p.R1188W
III/15	M	58	N	N	No PCLD	Normal
III/16	M	54	N	N	No PCLD	Normal
III/17	M	48	N	N	No PCLD	Normal
III/18 [1E]	F	53	Y	N	PCLD	p.R1188W
III/19	F	51	Y	N	PCLD	p.R1188W
III/20	F	48	N	N	No PCLD	Normal
III/21	F	52	N	N	No PCLD	Normal
III/22	M	49	N	N	No PCLD	p.R1188W
III/23	M	42	N	N	No PCLD	Normal
III/24	M	42	N	N	No PCLD	Normal
III/25	F	51	Y	N	PCLD	p.R1188W
III/26	M	50	N	Y	Renal cysts	p.R1188W
III/27	F	44	Y	Y	PCLD	p.R1188W
III/28	F	43	Y	N	PCLD	p.R1188W
III/29	M	51	Y	Y	PCLD	p.R1188W
III/30	F	47	Y	Y	PCLD	p.R1188W
III/31	F	46	N	N	No PCLD	Normal

Table S4. ***Continued***

Inclusion (n=40) PCLD-1	Sex	Age (y)	Liver phenotype (n=16)	Kidney phenotype (n=12)	Diagnosis (PCLD n=16; renal cyst(s) n=3)	*LRP5* Mutation (n=22)
III/32	F	48	N	N	No PCLD	Normal
III/33	M	47	N	N	No PCLD	Normal
III/34	M	45	N	N	No PCLD	Normal
<40 years						
IV/1	F	36	N	N	No PCLD	Normal
IV/2	M	34	N	N	No PCLD	Normal
IV/3	F	33	N	N	No PCLD	p.R1188W
IV/4	M	30	N	N	No PCLD	p.R1188W
IV/5	M	26	N	Y	Renal cyst	p.R1188W
Inclusion (n=14)	**Sex**	**Age (y)**	**Liver phenotype (n=6)**	**Kidney phenotype (n=2)**	**Diagnosis (PCLD n=6; ADPKD n=0)**	***LRP5* Mutation (n=6)**
PCLD-2						
I/1	M	75*	n.a.	n.a.	n.a.	n.a.
I/2	F	79*	n.a.	n.a.	n.a.	n.a.
II/1 (index)	F	86	Y	N	PCLD	p.V454M
II/3	M	83	N	N	No PCLD	Normal
II/4	F	77	N	N	No PCLD	Normal
II/5	M	67	N	N	No PCLD	Normal
III/1	F	49*	Y	Y (4 small cysts)	PCLD	p.V454M
PCLD-3						
I/1	M	67*	n.a.	n.a.	n.a.	n.a.
I/2	F	71	Y	N	PCLD	p.R1529S
II/1	F	48	Y	N	PCLD	p.R1529S
II/2 (index)	F	43	Y	N	PCLD	p.R1529S
II/3	F	40	N	N	No PCLD	Normal
PCLD-4						
I/1	M	73*	n.a.	n.a.	n.a.	n.a.
I/2	F	82*	n.a.	n.a.	n.a.	n.a.
II/1	M	70	N	N	No PCLD	Normal
II/2	M	67	N	N	No PCLD	Normal
II/3 (index)	M	65	Y	Y (3 cysts)	PCLD	p.D1551N
III/1	F	38	N	N	No PCLD	Normal
III/2	M	35	N	N	No PCLD	Normal

Abbreviations:
F, female); M, male); n.a., no data available; N, no cysts present; Y, yes, cysts present; * deceased and age of death; ** splenic cysts; [#] PCLD phenotype correlates with the ADPKD Ravine criteria (6, 7); [$] Radiological data resources ; [1E] Exome sequencing performance 1 in individual III/18; [2E] Exome sequencing performance 2 in individual II/18.

Additional text for Table S4.

We performed abdominal ultrasound screening of the liver and kidneys in 39 members of proband III/18 from the PCLD-1 family. Ultrasound images of liver and kidneys were acquired using a 3.6-MHz general-purpose clinical echo system (Acuson x150, Siemens AG) equipped with a curved linear array transducer. Presence or absence of cysts was carefully noted in all living relatives.

For almost all deceased relatives, DNA from blood or tissue and radiological imaging was not available. An abdominal CT scan for only one diseased patient (II/1) was present and showed a severe polycystic liver, but no DNA was available. Member III/6 died after inclusion during the research process. We restricted our inclusion to the five oldest members from the fouth generation of PCLD-1 family because of the late onset of hepatic cystogenesis (>40 years), especially in males. These members are recognized as individuals at risk for PCLD, which is associated with age-dependent occurrence and development of hepatic cysts.[7] Individual IV/5 was known with a large dominant renal cyst and his father (III/9) showed a severe polycystic liver and multiple cysts in both kidneys. Finally, 40 members were included for genotype analysis (excluding patient II/1).

Three males (II/13, III/22, III/26) were categorized as "No PCLD" because they did not fully met the Reynolds criteria for PCLD, even after reevaluation in II/13 and III/26 (See Supplementary Text).[7] Two males had bilateral renal cysts (and splenic cysts in individual II/13) with a positive family history of PCLD. All three members possessed the *LRP5 c.3562C>T* (p.R1188W) missense mutation. The mild or indeterminate phenotype in these members can be explained by the age-dependent factor for hepatic cyst formation, but even more by the predominant hepatic cystogenesis development in females and an evident known incomplete penetrance of ~80% in PCLD.[8, 9, 10]

There was no renal disease nor renal failure present in this PCLD family; however, 12 members possessed several renal cysts. Only patient III/9 was affected with numerous cysts in both kidneys closely associated to the ADPKD criteria.[4, 5]

Table S5. Location of mutations in LRP5 protein domains.

Family	Amino acid change	LRP5 domain	Location
PCLD-1	p.R1188W	4th β-propeller; 6th blade	Extracellular
PCLD-2	p.V454M	2nd β-propeller; 3th blade	Extracellular
PCLD-3	p.R1529S	Between PPPSP motif A and B	Intracellular
PCLD-4	p.D1551N	Between PPPSP motif B and C	Intracellular

Additional text for Table S5.

We identified four unique *LRP5* variants of which are in two located extracellularly and two are located intracellularly. The LRP5 protein structure was created by using a LRP6 template as start homology model and reconstruction by YASARA&WHAT-IF Twinset.[62] PDB files for homology modeling were available for four WD40 domains (β-propeller subdomains). Two *LRP5* variants are located at the second and fourth WD40 domains, respectively (Figure 1). Separate models for these domains and the mutations were visualized and analyzed using YASARA.

Table S6. Templates for homology models of the LRP5 protein domains.

Domain in LRP5	PDB-file	LRP5 identity (%)	Reference
1st WD40	3SOV	73	63
2nd WD40	3S94	79	64
3rd WD40	4A0P	80	65
4th WD40	4A0P	65	65

URLs

- Primer3, v.0.4.0 (latest version); http://frodo.wi.mit.edu/primer3/;[54]
- SNP Check, v.3 (latest version); a tool for performing batch checks for the presence of SNPs in predicted PCR primer binding sites; https://ngrl.manchester.ac.uk/SNPCheckV3/snpcheck.htm
- Human genome browser gateway; http://genome.ucsc.edu/cgi-bin/hgGateway; v.hg19 human reference genome (GCRh37);[50, 51]
- 1000 Genomes Project, a deep catalog of Human Variation; http://www.1000genomes.org/data#DataAccess (in 1,000 individuals variants were assessed in the project);[55]
- Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA; November 2012 accessed; http://evs.gs.washington.edu/EVS/ (in 6,500 individuals variants were assessed in the project);[24]
- Database of Single Nucleotide Polymorphisms (dbSNP); http://www.ncbi.nlm.nih.gov/projects/SNP/; Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine; NCBI dbSNP Build 137; 26th June 2012 available;[56]
- Mouse Genome Informatics; v.MGI 5.12 (last database update 03-13-2013); http://www.informatics.jax.org/searches/allele_report.cgi?_Marker_key=37359; MGD[57]
- Human gene mutation database (HGMD® Professional) (www.biobase-international.com/hgmd) from BIOBASE Corporation; Professional 2012.4, 14th December 2012 accessed;[53]
- MRS database; v.6 (latest version); http://mrs.cmbi.ru.nl/m6/entry?db=sprot&id=lrp5_human&rq=lrp5_human;[52]
- The Wnt homepage; 1997-2013 Roel Nusse; Last up-dated June 2013; http://www.stanford.edu/group/nusselab/cgi-bin/wnt/

CHAPTER 8

LRP5 VARIANTS MAY CONTRIBUTE TO ADPKD

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ABSTRACT

Mutations in *PKD1* or *PKD2* are causative for autosomal dominant polycystic kidney disease (ADPKD). However, a small subset of ADPKD probands do not harbor a mutation in any of the known genes. *LRP5* was recently associated with hepatic cystogenesis in isolated polycystic liver disease (PCLD).

Here, we demonstrate that this gene may also have a role in unlinked and sporadic ADPKD patients. In a cohort of 79 unrelated patients with adult-onset ADPKD, we identified a total of four different *LRP5* variants that were predicted to be pathogenic by *in silico* tools. One ADPKD patient has a positive family history for ADPKD and variant *LRP5 c.1680G>T* ; p.(Trp560Cys) segregated with the disease. Although also two *PKD1* variants probably affecting protein function were identified, luciferase activity assays presented for three *LRP5* variants significant decreased signal activation of canonical Wnt signaling.

This study contributes to the genetic spectrum of ADPKD. Introduction of the canonical Wnt signaling pathway provides new avenues for the study of the pathophysiology.

Keywords

Autosomal dominant polycystic kidney disease; Hepatic cysts; *LRP5*; *PKD1*; Sequencing

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD; MIM 173900) is the most common renal Mendelian disorder with a global prevalence of 1/400-1/1,000.[1] Development of multiple fluid-filled cysts may cause bilateral enlarged kidneys and result in end-stage renal disease (ESRD). Renal replacement therapy is necessary in about ~5% of ADPKD patients.[1] In addition, the majority of ADPKD patients have health care issues due to extrarenal features and complications such as polycystic liver disease (83%), hypertension (80%), cardiac valve abnormalities (25%), intracranial aneurysm (10%) and cysts in the pancreas and other organs.[2, 3]

There is a broad clinical expression of (extra-)renal manifestations and symptoms among ADPKD patients.[1,3] The phenotype of affected individuals from single large pedigrees may cover the complete spectrum of ADPKD ranging from few renal cysts to grossly enlarged polycystic kidneys with renal failure.[4] These observations suggest that modifying factors, *in trans* or multiple PKD mutations may influence the onset and course of renal cystogenesis.[5, 6]

The genotype-phenotype correlation is not completely understood in ADPKD.[7] Linkage studies identified two disease-causing genes, *PKD1* (Chr.16p13.3; NG_008617.1) and *PKD2* (Chr.4q22.1; NG_008604.1).[8, 9] Molecular screening may confirm the clinical diagnosis with a mutation frequency of 76% *PKD1* and 13% *PKD2*.[10, 11] Despite thorough screening ~11% of families remain unlinked to either locus, which suggest genetic heterogeneity.[10, 11] Approximately 10% of ADPKD patients have no family history of renal disease.[12] These solitary cases may have mild or severe polycystic kidneys due to incomplete disease penetrance, *de novo* (modifier) gene variants and environmental influences.

Recently, we identified the *LRP5* gene (Chr.11q13.2; NG_015835.1) as the third locus associated with isolated polycystic liver disease (PCLD; MIM 174050) by whole-exome sequencing in an extended family.[13] Since polycystic liver disease is the most common extra-renal feature in ADPKD patients, we hypothesized that *LRP5* variants may contribute to hepatic and renal disease heterogeneity in ADPKD. We addressed the role of *LRP5* by screening all 23 exons in 79 unlinked and/or sporadic ADPKD patients, and performed some functional analyses.

METHODS

Patients

This study was approved by the institutional review board and ethics committee of the Radboud university medical center, Nijmegen. All participants provided informed consent. The families were recruited in The Netherlands, screened according to the Ravine criteria[14] and blood samples were collected.

Polycystic Kidney Disease Mutation Analysis

Mutation screening of *PKD1* (NM_001009944.2) and *PKD2* (NM_000297.2) involved Sanger sequencing of exons and flanking intronic regions and multiplex ligation-dependent probe amplification analysis in all probands by using bidirectional Sanger sequencing on ABI3730 Genetic Analyzers (Applied Biosystems). *PKD1* variants were assessed using the Autosomal

Dominant Polycystic Kidney Disease mutation database v.3.0, http://pkdb.mayo.edu/.[15] *PRKCSH* (NM_002743.3) and *SEC63* (NM_007214.4) were analyzed in two probands with severe polycystic livers (109 from family A, and 101 from family D) and patient 102 (family B) was screened for *PKHD1* (NM_138694.3) and *HNF-1*β (NM_000458.2).

LRP5 Sequencing and *In Silico* Analysis

All 23 exons of the *LRP5* gene (NM_002335.3) were screened using High Resolution Melting followed by Sanger sequencing. Scoring of *LRP5* variants was performed with homology modeling, *in silico* analysis such as PolyPhen2, Mutpred, SIFT, Align GVGD, PhyloP and the Grantham score. Genome€-wide sequence data from the 1,000 Genomes Project, 6,500 individuals from the National Heart, Lung and Blood Institute Exome Sequencing Project (version 0.0.27), ~500 individuals from the Genome of The Netherlands, exome data from ~2,000 individuals of predominantly European ancestry sequenced in-house, and DNA samples from 525 Moroccan healthy, unrelated individuals served as controls.

We reported 1 *LRP5* variant (rs724159825) and 3 *PKD1* variants (rs724159824; rs724159822; rs724159823) described in this manuscript to the public dbSNP database.

Immunofluorescence and quantative PCR

Subsequently, immunofluorescence studies, luciferase activity assays and quantative PCR (qPCR) experiments with wild-type and four mutant *LRP5* constructs were conducted. Transfected HeLa cells were stained in order to elucidate the co-localization of LRP5 to the endoplasmic reticulum, Golgi apparatus and the plasma membrane (Supplementary Methods).

Luciferase Activity Assay and Real-Time PCR

Wnt signaling activity was assessed using the Cignal Reporter TCF/LEF Assay Kit (Qiagen) after transfecting CHO or HEK293 cells with wild-type or mutant constructs and addition of hWnt3a (R&D Systems).

Expression levels of Wnt target genes were assessed in Wnt3a-activated and non-activated transfected HEK293 cells by Real-Time PCR (RT-PCR).

RESULTS

A subset of 29 ADPKD patients had genotypic prescreening. Molecular diagnostics was performed for *PKD1*, *PKD2* and *PKHD1* (Chr.6p12.2; NG_008753.1) in adults. Additional analysis of *HNF1ß* (Chr.17q12; NG_013019.1) was performed in adolescents when genotype screening of *PKD1*, *PKD2* and *PKHD1* were negative. No point mutations, CNV or large deletions were identified and all 29 affected individuals were designated as being unlinked. A group of 50 patients had a clinical diagnosis of ADPKD with a symptomatic polycystic liver. Variants for PCLD in *PRKCSH* (Chr.19p13.2; NG_009300.1) and *SEC63* (Chr.6q21; NG_008270.1) affecting protein function were excluded, but the PKD genotype was unknown in this group. In this heterogeneous cohort of 79 ADPKD patients, we identified four *LRP5* variants by Sanger sequencing (Table 1). Both unique *LRP5* variants were detected in the subgroup of ADPKD

Table 1. Patient characteristics.

Baseline characteristics				At first presentation (diagnosis)			Treatment for polycystic kidneys
Family	Subject	Age (y)	Sex	Age (y)	Creatinin (µmol/l)	ESRD	Medical/ Surgical
A	109	56	F	51	155	Yes	Thiazide and AT2-receptor antagonist; 9-months octreotide
	202	33	F	24	79	No	Antihypertensive treatment
B	101	60	M	55	80	No	Antihypertensive treatment
C	102	18	F	14	64	No	-
D	101	51	F	43	114	Yes	Antihypertensive treatment; kidney transplantation

with a severe polycystic liver. Here we present the data of 3 adult-onset ADPKD patients and 1 adolescent ADPKD patient (Figure 1 and Figures S1, S2 and S3).

Family A

The proband (109) presented to us with a symptomatic polycystic liver and renal cysts of various sizes at 51-years-of-age (Table 1). She was clinically diagnosed as ADPKD. Mutational screening of *PRKCSH* and *SEC63* yielded no variants causing hepatic and renal cystogenesis. She was from Moroccan ancestry and her ancestors did not have renal disease suggestive for ADPKD. During the first pregnancy of her oldest daughter (202), polycystic kidneys and several small hepatic cysts were detected by abdominal ultrasonography. Renal failure was absent and mild hypertension was under control by use of an AT2-receptor antagonist. Both patients fulfilled the unified Ravine criteria for ADPKD.[14]

By *LRP5* variant analysis, we identified a unique missense variant *c.1680G>T* ; (p.(Trp560Cys); rs377144001) in both Moroccan patients (109 and 202). This *LRP5* variant is not detected in genome-wide sequence data such as 1,000 Genomes Project, and ~2,000 in-house exome sequencing data from patients of European ancestry. In addition, *LRP5 c.1680G>T* is neither detected in 4,400 African-American exome sequencing cohort from the National Heart, Lung and Blood Institute (NHLBI) Exome Sequencing Project (ESP). This variant was recently identified once in an ESP European-American cohort with a total allele frequency of 1/12,988 (rs377144001, dbSNP v.138). The variant *LRP5 c.1680G>T* was not present among 525 tested Moroccan DNAs of unrelated individuals.

In silico analysis predicts this variant to be pathogenic and alters a conserved LRP5 amino acid (Table 2). Homology modeling indicated that this missense variant affects one of the tryptophans which are important for the core structure of this domain. Multiple hydrophobic interactions and a hydrogen bond will be lost by the tryptophane amino acid change into cysteine. This variant is highly likely to change the secondary and tertiary structure of the protein, and to disturb the function of the second WD40 domain function.

Variants in the genes *PKD2*, *PKHD1*, *PRKCSH* and *SEC63* affecting the protein function were absent, but molecular diagnostics of *PKD1* revealed two unique variants. Both *PKD1* variants, *c.1281_1283delGGC* ; p.(Ala428del) (rs724159824) in exon 6 and *c.3133G>C* ; p.(Val1045Leu) (rs724159822) in exon 13, were present in the proband (109). The p.(Ala428del) had been transmitted to the 33-year-old affected daughter (202), while the 27-year-old healthy daughter (203) inherited p.(Val1045Leu). In addition, the 25-year-old healthy daughter (204) harbors also p.(Val1045Leu), but in a homozygous state (*PKD1 c.3133G>C* homozygous). Both *PKD1* variants are unreported in the literature (unclassified variants), online and in-house databases and are localized in evolutionarily moderately-low conserved domains.

The *PKD1* missense variant p.(Val1045Leu) may be a benign variant since this variant had been transmitted to the unaffected daughter (203). The consequence of this *PKD1* variant is currently unclear, but non-penetrance or late-onset of ADPKD may play a role. The contributing effect of p.(Ala428del) is unclear, but homology modeling suggests that the nascent residues surrounding codon 248 are not conserved and that the C-type lectin domain is not pathogenic altered. It is well conceivable that the identified changes in this patient may exhibit their full pathogenic effect in concert.

Family B

A 57-year-old Dutch male came to our attention because of uncontrolled hypertension. Extensive clinical and radiological examination demonstrated large, complicated (multilocular) renal cysts (Figure S1).

There was no family history for polycystic diseases, liver or renal disease. Therefore, no consent was retrieved for phenotype and genotype screening in family members. *PKD1* and *PKD2* variants were absent in the proband and Sanger sequencing of the *LRP5* gene identified *c.3107G>A* ; p.(Arg1036Gln; rs61889560). This variant reported an incidence of 0.001-0.005

Table 2. *LRP5* variants in ADPKD patients.

Family	Ethnicity	Position (GRCh37; hg19)	Variant (*c.DNA*; NM_002335.2)	Exon (NG_015835.1)	Predicted effect on protein
A	Moroccan	g.68171046	*c.1680G>T*	8	p.(Trp560Cys)
B	Dutch	g.68191036	*c.3107G>A*	14	p.(Arg1036Gln)
C	Dutch	g.68192736	*c.3403C>T*	15	p.(Arg1135Cys)
D	Dutch	g.68193486	*c.3468G>C*	16	p.(Gln1156His)

*Allele frequency

(1/245) in public databases. The missense variant is located at the fourth WD40 and may lead to a less stabilized domain due to loss of hydrogen and ion binding.

Family C

A 14-year-old female (102) was diagnosed with 2 large, symptomatic cysts with a diameter of 4.5 cm each and a small ('intrapolar') cyst were present in the left kidney with a normal right kidney (Figure S2). Creatinine levels remained between 61-71 µmol/l within the normal range (50-90 µmol/l) during 4 years of follow-up. Her blood pressure was 130/70 mmHg and regularly monitored at home. Family history was negative for ADPKD, ARPKD and PCLD or other genomic disorders, but her mother was diagnosed at the age of 30 with nephrotic syndrome of unknown cause. Both parents were screened and there were no structural renal abnormalities on ultrasonography.

Molecular diagnostics showed no evidence for the diagnosis of ADPKD or ARPKD by analyses of the *PKD1*, *PKD2*, *PKHD1* and *HNF1*β gene. We identified the *LRP5* variant *c.3403C>T* ; p.(Arg1135Cys; rs143396225). This variant is reported with a frequency of 1/1,300 in the European American ESP cohort from the NHLBI, and absent in the 1,000 Genomes Project dataset and ~2,000 DNA exomes sequenced in-house. *In silico* analyses predicted *LRP5 c.3403C>T* to be disease-associated with pathogenic effects for the protein function (Table 2). Homology modeling showed that the arginine is located at the fourth WD40 domain. The p.(Arg1135Cys) variant results in a change of a hydrophilic molecule to an apolar hydrophobic amino acid which may contribute to a destabilized WD40 domain. The loss of ionic interactions is likely to affect the function of the protein domain.

Family D

Bilateral enlarged kidneys containing multiple fluid-filled cysts were an accidental finding on ultrasonography in the proband (101) at the age of 43 (Figure S3). Her blood pressure was

Polyphen2 (Class; score)	Mutpred	SIFT (Class; score)	Align GVGD	PhyloP	Grantham score	Frequency in controls* (EVS)
Probably damaging (1.00)	0.770	Deleterious (0.00)	Class C65 (GV:0.00-GD:214.36)	5.54	215	1/12,988
Possibly damaging (0.658)	0.785	Tolerated (0.06)	Class C0 (GV:28.82-GD:19.90)	1.74	42	1/245
Probably damaging (1.00)	0.586	Deleterious (0.00)	Class C65 (GV:0.00-GD:179.53)	4.00	180	1/1,299
Probably damaging (1.00)	0.702	Deleterious (0.00)	Class C15 (GV:0.00-GD:24.08)	3.76	24	-

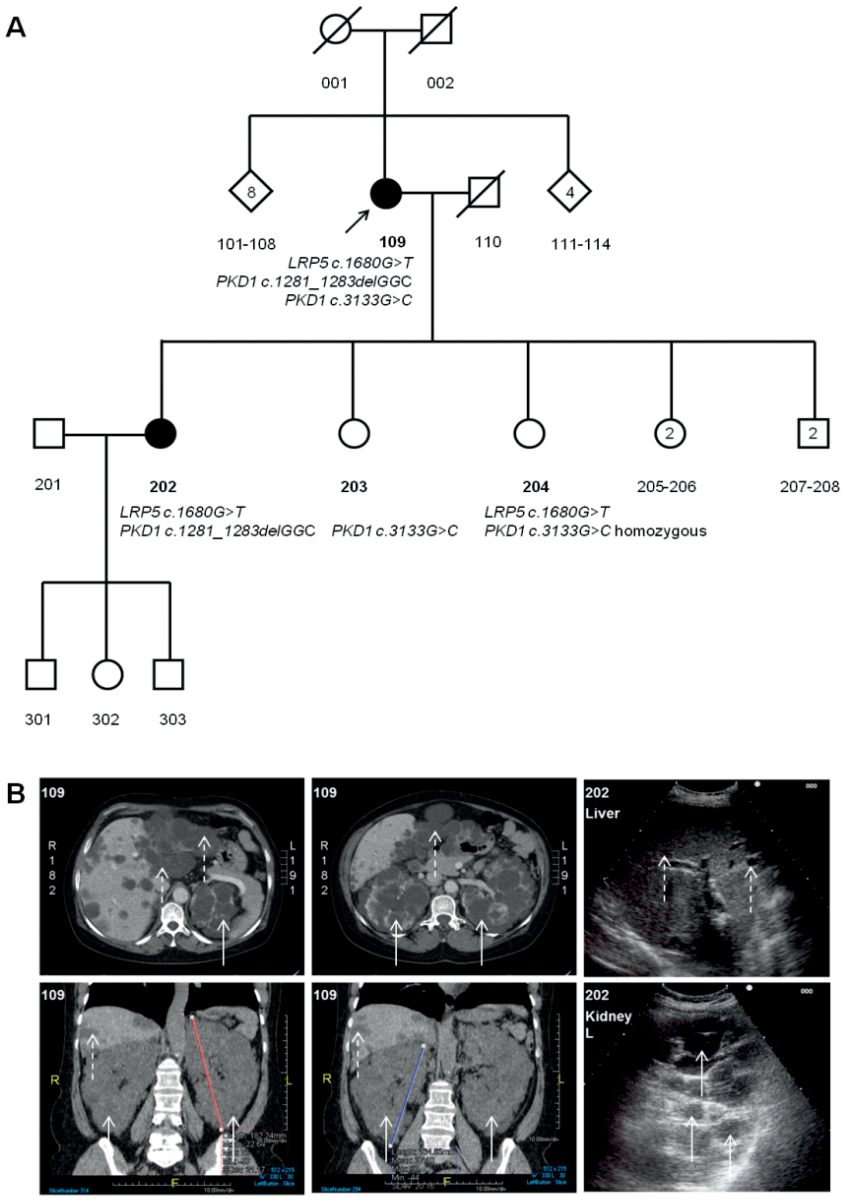
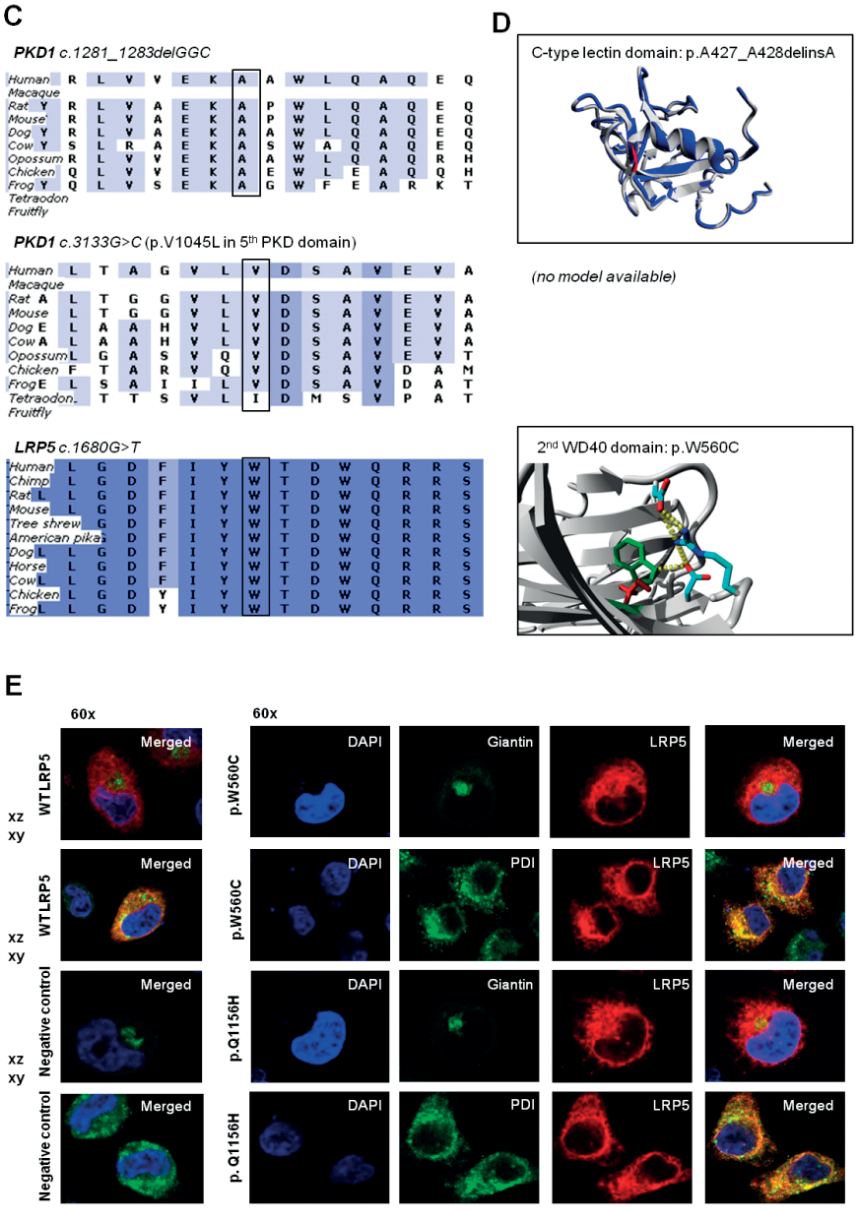


Figure 1. Clinical and genetic data of ADPKD family A. Pedigree of family A present two affected individuals, proband 109 and daughter 202. Affected individuals indicate those with confirmed ADPKD on CT scanning or abdominal ultrasound according to the Ravine criteria. (B) CT scanning in proband 109 presented a severe polycystic liver in segments 2, 3 and 8 and the kidney sizes were craniocaudal 19cm (left) and 21cm (right) in diameter. Abdominal ultrasonography in patient 202 assessed polycystic kidneys (white arrows) and multiple small hepatic cysts (dotted white arrows). (C) *PKD1 c.1281_1283delGGC* and *PKD1 c.3133G>C* were detected in the proband. Both *PKD1* variants are located in low evolutionary conserved regions in contrast to *LRP5 c.1680C>T*. ▶



▶(D) The in-frame *PKD1* deletion is located on an extracellular C-type lectin domain at the end of a loop close to a double helix structure. The alanine deletion shortens the strand, but there is no destabilization of the domain which is closely located to a rigid double helix structure with many hydrogen bounds. Missense variant *PKD1* p.(Val1045Leu) is located on the fifth tandem PKD domain without a difference in charge between wild-type and mutant protein. (E) Confocal imaging studies in transiently transfected HeLa cells identified co-localization of LRP5 in the endoplasmic reticulum. There were no differences between LRP5 localization compared to the wild-type *LRP5* construct for all four *LRP5* variants.

under control (128/70 mmHg) with an antihypertensive ACE-inhibitor and she used antacid medication. Eight years after identification of polycystic kidneys, the 51-year-old patient received renal transplant from a living unrelated donor.

Family history for polycystic or renal diseases was unremarkable. No *PRKCSH* or *SEC63* variants affecting the protein function were present, but *PKD* screening revealed 2 *PKD1* variants; *c.8293C>T* ; p.(R2765C) (rs144979397) on exon 23 and *PKD1 c.11554delC* ; p.(L3852Wfs*93) (rs724159823) on exon 42. The mother also harbors the *PKD1* missense variant p.(R2765C) which is predicted to be likely hypomorphic.[30,15, 16] Frameshift variant *PKD1 c.11554delC* is a recently reported variant with a clear pathogenic character.[16] *PKD1* analysis in genomic DNA from the father was unavailable.

Sequence analysis of the *LRP5* gene yielded a unique *LRP5 c.3468G>C* ; p.(Gln1156His) (rs724159825) in the proband which was absent in the DNA from both parents. The *LRP5* variant p.(Gln1156His) was absent in dbSNP and all aforementioned online and in-house exome sequence datasets. This missense variant was predicted to be damaging and the amino acid is located between blades 5 and 6 of the fourth β-propeller domain. Glutamine may interact by hydrogen bonds with adjacent arginine, but shows hydrophobic features at this position. Histidine contains an imidazole functional group which stereometrically changes the position. The major effect of this missense variant is the change in electrostatic charge which may result in disturbed interactions at the surface of the protein domain.

Functional studies

Confocal imaging studies identified co-localization of LRP5 in the endoplasmic reticulum, but no differences were identified between LRP5 localization compared to the wild-type *LRP5* construct for all four *LRP5* variants (Figure 1).

Luciferase activity assays of variant *LRP5* compared to wild type *LRP5* constructs were conducted to estimate Wnt signaling defects. Addition of ligand Wnt3a reduced Wnt signaling activity in *LRP5* variants p.(Trp560Cys), p.(Arg1036Gln) and p.(Gln1156His) (Figure 2). Wnt signaling activation showed an increasing tendency for *AXIN2* and *LEF1* gene expression (supplementary figures). *LRP5* variant p.(Trp560Cys) compared to *LRP5* wild-type presented significant increased *AXIN1* and *LEF1* expression by Wnt3a activation.

DISCUSSION

The data presented here suggest a role of *LRP5* in ADPKD. We discovered two unique and two rare *LRP5* variants in ADPKD patients regardless of family history or presence of *PKD* mutations. The association of *LRP5* variants with hepatic cystogenesis was identified in an extended PCLD pedigree.[13] The fact that polycystic livers are present in up to 94% of ADPKD patients[2] prompted us to investigate the possible pathogenic role of *LRP5* variants in ADPKD patients. There are a number of functional and animal studies that support the thinking that *LRP5* variants may contribute to hepatic and renal cystogenesis.

LRP5 is a single transmembrane co-receptor involved in the canonical Wnt signaling pathway that orchestrates mammalian organogenesis. Northern blotting studies indicate that LRP5 is strongly expressed in both liver and kidneys.[17] In addition, there is abundant LRP5 expression

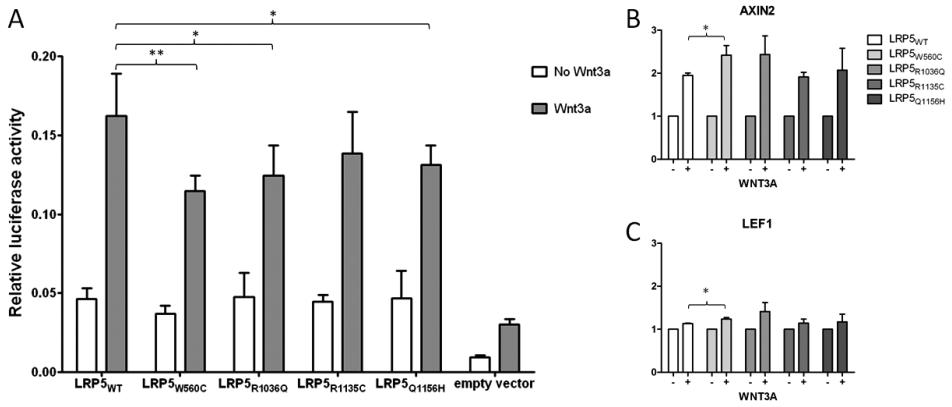


Figure 2. *LRP5* variants present reduced activation of canonical Wnt signaling. Canonical Wnt signaling activity was analyzed by firefly luciferase activity and normalized to renilla luciferase activity without (white bar) and with (grey bar) addition of 250 ng/mLWnt3a. *LRP5* constructs showed a significant increase in Wnt signaling activity compared with the empty vector. *LRP5* variants p.(Trp560Cys), p.(Arg1036Gln) and p.(Gln1156His) presented a decreased Wnt3a-induced signal activity (*p<0.05; **p<0.01). (B) All *LRP5* variants indicated only concordantly increased *AXIN2* expression levels by Wnt3a activation. (C) *LEF1* and others showed slightly increased gene expression. Significant increased *AXIN1* and *LEF1* expression by Wnt3a activation was present in *LRP5* variant p.(Trp560Cys) compared to *LRP5* wild-type (*p<0.05). The y-axis presents the relative gene expression level.

in convoluted tubules of human and mouse kidneys.[18] Canonical Wnt signaling is established by binding of a Wnt ligand to a cognate Frizzled receptor in the presence of LRP5. The role of canonical Wnt signaling in ADPKD has been an area of research.[19-24]

Increased canonical Wnt signaling[19-21] and diminished signal activation or stable β-catenin[22-24] that lead to an opposite effect of planar cell polarity/non-canonical signaling (PCP) have been reported in different ADPKD mouse models. These data suggests renal cystogenesis is initiated by imbalance between canonical and PCP signaling.[25] Indeed, polycystins modulate Wnt activity and disruption of either pathway may lead to polycystic kidney disease.[19, 20] During the late stage of nephron maturation polycystins are required for terminal differentiation of epithelial cells.[26] This led to the model that put the canonical/β-catenin pathway as one of the downstream effectors for ADPKD.[27]

A targeted knock-out *LRP5* mouse develops hepatobiliary abnormalities without specified hepatic nor renal morphology[23], although it is known that *LRP5* models have limited severity of Wnt disease phenotype compared to the almost identical *LRP6* gene.[28] The *LRP6* knock-out mouse model is lethal, in view of severe developmental defects that affect skeleton, retina, ureter (E8.5). At E18.5 macroscopic small cystic kidneys are visible, suggesting a PKD phenotype.[23] These findings support the notion that Wnt signaling is pivotal during early kidney development while polycystins appear to be critical during final stages of renal tubulogenesis.[27]

This paper provides evidence that the discovered variants affect LRP5 function. Luciferase activity assays of four *LRP5* constructs decrease Wnt3a-induced signal activation across various cell lines (Figure 2).[13] In addition, the expression of *AXIN2*, a Wnt signaling gene is enhanced in

the presence of 3 *LRP5* mutants (Figure S5). This suggests that there is constitutive activation of the Wnt signaling as a result of these *LRP5* variants.

Loss or gain of function of genes involved in Wnt signaling may lead to an imbalance between canonical and non-canonical signaling events.[29] There is evidence that key players of Wnt signaling modulate the renal disease severity in ADPKD by antagonizing canonical Wnt signaling.[5, 6] Three SNPs in *DKK3* downregulate canonical Wnt signaling similar to our data for *LRP5*.[5] In addition, *HNF-1*β which orchestrates Wnt signaling may aggravate congenital renal cystogenesis in mice and human ARPKD and ADPKD.[6, 30] Failure of canonical Wnt signaling homeostasis will result in activated non-canonical signaling. It is likely that other genes or modifier genes in the Wnt signaling pathway are linked to hepatic and renal cystogenesis.[19]

Multiple *PKD* variants may cause early and more severe renal disease in ADPKD.[6] Although the pathogenic character of both *PKD1* variants in family A remains unclear, we surmise that the resulting in-frame deletion of an alanine amino acid neighboring another alanine does not significantly affect the protein domain structure. A *PKD1* missense variant p.(Val1045Met) (*c.3133G>A*) in the fifth PKD domain has been predicted to be likely hypomorphic.[31] At the similar sequence position we detected *PKD1 c.3133G>C* leading to a hydrophobic amino acid change. Leucine and methionine are different regarding presence of a methyl group in leucine and a sulfide molecule in methonine which are both larger components compared to amino acid valine.

The case is different for the *PKD1* variants identified in proband 101 from family D. *PKD1 c.8293C>T* ; p.(Arg2765Cys) is present in an unaffected parent, but also probably hypomorphic and is weakly conserved.[15, 32] The second *PKD1* variant, *c.11554delC* ; p.(Leu3852Trpfs*93), is detected by next-generation sequencing and probably causative for the ADPKD phenotype.[16] This family had a negative history for ADPKD which suggests a *de novo* onset of the *PKD1* gene variant which has been described before.[12] In order to detect the additional mutational effect of a *LRP5* variant to the *PKD1 c.1154delC*, investigation of the severity of clinical ADPKD phenotype and subsequent functional studies are required.

Previous studies provided evidence that the type of *PKD1* variant correlates strongly with renal survival. Carriers of a truncating *PKD1* variant have an earlier age at onset of ESRD which supports the concept of a genotype phenotype correlation in ADPKD.[33]

In conclusion, several *in vitro* and *in vivo* studies suggest involvement of aberrant Wnt signaling and unregulated β-catenin levels contribute to renal cystogenesis.[22, 23] Our findings are in line with the hypothesis that genes encoding Wnt/β-catenin signaling partners may be candidate loci for cystic disorders.[13, 19] We postulate that *LRP5* variants may render ADPKD patients more susceptible to the development of polycystic liver although further studies are needed to validate our findings.

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SUPPLEMENTARY TEXT

Additional Information ADPKD Families

Family A

Family history

The proband (109) was the ninth child from a family of 13 from Moroccan ancestry. Almost all relatives emigrated to different countries in Europe and Africa, and therefore they had scarcely any familial contact. Her father died at the age of 53 years of an unknown cause. Her mother reached the age of 91 years. Both parents were unknown for PCLD, ADPKD, and no renal diseases or abdominal complaints are reported. The initial family history was negative for polycystic liver and kidney diseases. To our knowledge now, possibly one cousin (~40-year-old nephew of 109) is known with hepatic cysts and referred for surgery in Morocco (meta-history). During the pregnancy of her oldest daughter (202), renal cystogenesis was detected by ultrasound screening.

Proband 109

The 56-year-old proband 109 from Moroccan ancestry was affected with multiple cysts spread throughout the liver and both kidneys. It remains unknown when the first diagnosis of ADPKD is established. Our information reaches until the moment she visited a secondary hospital ~5 years ago. There, she was clinically diagnosed as ADPKD patient according to the Ravine criteria.

When she visited our hospital, renal failure was present with MDRD-GFR 24 ml/min/1.73m^2 (creatinin 188 µmol/l) at the age of 56-years-old. She had a history of H. bacter gastritis, short-term octreotide treatment (9 months). There was no indication for liver transplantation after consultation of a pre-liver transplant institution. Contraceptive medication had been administered during 20 years and she had seven pregnancies. She had major complaints due to the volume effect of multiple cysts. Superficially liver and kidney cysts were palpable during clinical examination and caused daily abdominal tenderness and pain. Furthermore, she complained of dyspnea, nausea, pyrosis and fatigue. The condition of proband 109 was not sufficient to enroll a clinical trial as treatment strategy. No renal replacement therapy has been started. Currently, renal function and hypertension are under control three-monthly in a secondary care hospital (Figure 1).

Family member 202

Clinically, only her oldest daughter (202) presented several small hepatic cysts and bilaterally polycystic and enlarged kidneys. Renal failure is absent and she has no renal replacement therapy. She delivered three healthy children and used anticonceptive medication for several (about three) years until now. Recently, she had two times treatment for proven pyelonephritis. Her blood pressure is under control with an anti-hypertensive AT2-receptor antagonist.

Family members 203 and 204

We were able to contact two more daughters. After informed consent, we performed abdominal ultrasound screening of the liver and both kidneys. Family member 203 was a 27-year-old female without a medical history. Family member 203 was a 25-year-old female without a medical history also. They had no symptoms associated with liver or renal disease nor used any medication (Table S1 and S2). No hepatic or renal cysts were detected by using a 3.6MHz general-purpose clinical echo system (Acuson x150, Siemens AG, Germany) equipped with a curved linear array transducer.

Genotype analysis

In our hospital, we previously performed molecular diagnostics for *PRKCSH* and *SEC63*. We detected no pathogenic variants associated with PCLD.

Screening of all 23 *LRP5* exons identified a unique variant *c.1680C>T* ; p.(Trp560Cys) (Table 1 and S3). Molecular diagnostics of the *PKD1* gene revealed 2 private *PKD1* variants, *c.1281_1283delGGC* (p.(Ala428del)) and *c.3133G>C* (p.(Val1045Leu)) in the proband (109). The p.Ala428del had been transmitted to the 33-year-old affected daughter (202), while the 27-year-old healthy daughter inherited p.(Val1045Leu) (205). Both *PKD1* variants have not been reported in the literature, online and in-house databases before and are localized in evolutionarily moderately-low conserved domains. The *PKD1* missense variant p.(Val1045Leu) can be regarded as a benign variant since this variant had been transmitted to the unaffected daughter. However, the exact character of the p.(Ala248delinsAla) is unclear (Table S4 and S5). Analysis of homology models demonstrated that the nascent residues surrounding codon 248 are not conserved and that the C-type lectin domain is not pathogenic altered (Table S6 and S7). In addition, only one *PKD1* deletion (*c.1273_1275delGAG*; p.(Glu425del)) located on exon 6 has been described.[15]

We identified the missense variant *c.1680G>T* ; p.(Trp560Cys) in the *LRP5* gene and confirmed presence of this variant in an 33-year-old affected child (202) using Sanger sequencing.

Family B

Family history

There was no apparent family history for polycystic diseases, renal or hepatic diseases. The family origins from The Netherlands and present no familial disorders. Thirty-five years ago, the father of proband 101 deceased at the age of 66 because of colon cancer (Figure S1). His mother reached the age of 84 and died due to age. Additional inquires for hepatic or renal (cystic) disease was negative in brothers, sisters and cousins.

Proband

This 60-year-old male proband had no history of polycystic diseases, renal or hepatic disease nor ADPKD-related extra-renal manifestations before polycystic kidneys were detected. Two years after the clinical diagnosis of ADPKD, the patient developed paroxysmal atrial fibrillation, neutropenia e causa ignota and scarce urolithiasis. He administered a HMG-CoA

reductase inhibitor (statin), a combined antihypertensivum with an ACE-inhibitor and a calcium antagonist (coveram), a β-blocker, thiazide and aspirin. The blood pressure is under control and the patient presents no symptoms.

Genotype analysis
Unfortunately, there was no DNA from both parents available. Parentage screening of *LRP5 c.3107G>A* could not be conducted.

Family C

Family history
No apparent family history was present for hepatic and/or renal disease, including polycystic diseases (Figure S2). Both parents had screening for cystogenesis when proband 202 was diagnosed with renal cysts. The mother and father presented normal kidneys and urinary system. No consent was provided to perform abdominal ultrasound screening for the liver and both kidneys. Only two cysts in the right adnex was detected in the mother. Additional family studies were not indicated. The history of the father of proband 202 presented an episode of liver function disturbances *e causa ignota*. Abdominal ultrasonography excluded steatosis and intrahepatic bile duct dilatations, and the liver function resolved spontaneously.

Proband
Clinical examination indicated no liver disease which was confirmed by normal liver test values and MRI scan. She suffered from episodes of nausea and initially left-sided abdominal pain from unknown origin. The abdominal pain was frequently associated with vomiting and sustained after different treatment strategies. She presented non-dysmorphic features and additional clinical investigations of the heart, lung, extremities and development were unremarkable. Abdominal ultrasonography revealed unilateral renal cystogenesis, but no dysgenesis of the right kidney. Finally, this was the explanation for her abdominal complaints. Renography excluded ureteropelvic junction stenosis and presented 44% function of the left kidney and 56% of the right kidney. Recent radiological examination presented regular renal contour, normal cortex and medulla differentiation of both kidneys. The length of the left and right kidney was assessed at 13.4 cm and 11.7 cm respectively without development of novel renal cysts or other urinary tract anomalies. In addition, multicystistic ovaria were detected on ultrasonography. The patient is normotensive.
Genotype analysis
Molecular diagnostics for *PKD1*, *PKD2*, *PKHD1* and *HNF1*β were negative in the proband. The only detected variant is *LRP5 c.3403C>T* ; p.(Arg1135Cys).

Family D

Family history
Her father died at the age of 70 from metastasized urothelial carcinoma grade III. CT scanning showed normal liver and kidneys at that time. Her 75-year-old mother had hypertension and

suffered from vascular diseases. Proband 101 has no brothers or sisters. No consent was given for screening of her 18-year-old son. In addition, no apparent extra-renal ADPKD-associated manifestations were present by taking history.

Proband

Polycystic kidneys were identified in this index patient at the age of 43. The first sign was abdominal swelling. She administered oral contraconceptives for 15 years and had one pregnancy. Four years after diagnosis was set she presented to us with pyrosis, fatigue, weight loss and anorectic problems (Figure S3). Major complaints included frequent right-sided pain attacks for 2-3 days and a decreased physical activity. Hepatomegaly and cysts were palpable in both flanks by clinical examination. Multiple hepatic cysts and polycystic kidneys were detected by abdominal ultrasonography. Her blood pressure was under control (128/70 mmHg) with antihypertensive ACE-inhibitor and administered anti-acida. At presentation a pre-terminal renal insufficiency with GFR 21 ml/min/1.73m^2 (creatinin 114 µmol/l) with normal liver parameters was assessed and renal function declined within 3 years. Patient 101 required a kidney donor and underwent pre-transplantation screening when her creatinin level was increased to 297 µmol/l in 2009. She underwent a living kidney donor transplantation at the age of 51.

Genotype analysis

LRP5 c.3468C>G ; p.(Gln1156His) was detected in the proband. Although her father deceased 13 years earlier, we were able to isolate DNA from paraffin-embedded tissue. CT scans before his death showed no cystogenesis.

The living kidney transplantation for proband 101 derived from an unrelated, healthy individual. *PKD* screening revealed two *PKD1* variants; *c.8293C>T* (p.(Arg2765Cys); exon 23) and *PKD1 c.11554delC* (p.(Leu3852Trpfs*93); exon 42). The mother also harbors the *PKD1* missense variant (rs144979397) which is predicted to be likely hypomorphic. Frameshift variant *PKD1 c.11554delC* is a recently reported variant with a clear pathogenic character (Table S6 and S7). *PKD* analysis in the father was not possible because genomic DNA was unavailable.

SUPPLEMENTARY METHODS

DNA Isolation

Genomic DNA was extracted from blood leukocytes using the HP-PCR Template Preparation kit (Roche Applied Science). DNA isolation from paraffin-embedded sections was performed in individual 002 from family D using the QIAamp DNA Micro-Kit (Qiagen).

LRP5 Variant Detection

For all 23 *LRP5* coding exons primers were designed using Primer3 software.[34] We performed high resolution melting curve analysis using the RotorGene-Q and ScreenClust software (Qiagen) and validated genotype variants by Sanger sequencing on ABI3730 Genetic Analyzers (Applied Biosystems) (GRCh37, hg19).[35, 36] *LRP5* primer sequences are available on request.

LRP5 Variant Analysis

We created the LRP5 protein structure using a LRP6 template as start homology model and reconstruction by YASARA&WHAT-IF Twinset.[37] For WD40 domains (β-propeller subdomains) PDB-files were available to incorporate the identified *LRP5* variants for analysis of structural effects.[38, 39]

In silico analysis with PolyPhen2, Mutpred, SIFT, Align GVGD, PhyloP and the Grantham score.

Genome-wide sequence data from the 1,000 Genomes Project[40], 6,500 individuals from the National Heart, Lung, and Blood Institute Exome Sequencing Project (EVS Release Version: v.0.0.27., April 18, 2014)[41], ~500 Dutch individuals from the Genome of The Netherlands[42], and exome data from 2,000 individuals of predominantly European ancestry sequenced in-house served as controls.[43, 44]

DNA samples from 525 Moroccan healthy, unrelated individuals were used as controls for *LRP5 c.1680G>T* ; p.(Trp560Cys) in family A from Moroccan ancestry.

Immunofluorescence Studies

$5.0x10^4$ HeLa cells per well were seeded on poly-L-lysine coated Ø12mm cover glasses in a 24-wells plate and transiently transfected with 500ng of wild-type or mutant *LRP5* construct. After 24 hours medium was refreshed and cells were cultured for another 24 hours followed by paraformaldehyde fixation and antibody immunofluorescence staining.

Antibodies

A rabbit monoclonal anti-LRP5 antibody (Clone D23F7; 1:200; Cell Signaling) recognizing residues surrounding Pro1527 of human LRP5 protein (#3889; 200kDa) was conducted for imaging studies.

For immunofluorescence assays rabbit anti-LRP5 was combined subsequently with primary antibodies against endoplasmic reticulum, golgi and cell membrane proteins. The following primary antibodies were used mouse monoclonal anti-protein-disulfide isomerase (PDI) (Clone 1D3; 1:500; Stressgene Bioreagens), mouse monoclonal anti-giantin (Clone G1/133; 1:100; Enzo Life Sciences), mouse monoclonal anti-CD44 (Clone 15-3c11; 1:400; Thermo scientific). Secondary AlexaFluor-conjugated antibody immunostaining (Alexa488, Alexa568; 1:200 and Alexa647; 1:100; Invitrogen) and 4',6-diamidine-2-phenylindole (DAPI) nucleus staining (1µg/ml Sigma) were used for immunofluorescence image acquisition by a high content microscope (Leica TCS SP5 Microsystems) and a confocal laser scanning microscope (Fluoview FV1000, Olympus).

Mammalian Expression Constructs

Total RNA was isolated from liver tissue using Trizol Reagent (Invitrogen) and oligodT cDNA was obtained by RT Transcriptor First Strand cDNA synthesis kit (Roche Applied Sciences). Full length wild-type LRP5 was obtained using the Faststart High Fidelity PCR System (Roche). LRP5 was cloned into the mammalian expression vector pcDNA3.1V5His TOPO-TA (Invitrogen) and checked by sequence analysis. *LRP5* mutants *c.1680G>T*, *c.3107G>A*, *c.3403C>T* and *c.*

3468C>G were constructed by mutating the pcDNA.LRP5.WT vector using the Quick Change-II-XL Site-Directed Mutagenesis Kit (Agilent Technologies). Primers for *LRP5* constructs are available on request.

Luciferase Acitivity Assay

For the activity assay $5.0x10^3$ CHO cells per well were seeded in a 96-wells plate in triplicates and cultured them for 24 hours. Cells were transiently transfected using X-tremeGeneHD (Roche) with 100ng *LRP5* construct or empty vector and 100ng of Reporter or 100ng Negative control (Cignal Reporter TCF/LEF Assay Kit; Qiagen). After 16h cells were washed with PBS and medium with or without 250ng/ml hWnt3a (5036-WN, R&D Systems) was added to initiate the Wnt signaling. Cells were cultured for another 24 hours and luciferase activity was detected using the Dual-Glo Luciferase assay (Promega) in a TECAN M200 plate reader. Firefly luciferase activity was normalized to Renilla luciferase activity for variations in transfection efficiencies as reported previously.[13] These experiments were repeated in HEK293 cells and presented identical results for $LRP5_{R1135C}$ and similar significant decreased activated signaling for $LRP5_{W560C}$ and $LRP5_{R1156Q}$. Transiently transfected CHO and HEK293 cells expressed >1,000 times more compared to the empty vector. These experiments were conducted in triplicates and performed three times and values present means ± standard deviation.

Quantative PCR Experiments

We conducted transient transfections of human embryonal kidney cells (HEK293; ATCC CRL-1573) with *LRP5* constructs as previously described.[13] The signaling was activated by addition of Wnt3a for 24 hours. Total RNA was extracted with TRIzol (Invitrogen). Template cDNA was obtained using the iScript cDNA synthesis kit (Biorad). Expression levels of Wnt target genes were assessed twice by qPCR experiments (in triplicates). We researched genes associated with the canonical Wnt signaling pathway listed at the Wnt homepage; *axis inhibitor-1* (*AXIN-1*), *axis inhibitor-2* (*AXIN-2*), *cyclin D1* (*CCND1*), *lymphoid enhancer-binding factor 1* (*LEF1*) and other target genes.

The transfected cells expressed LRP5 >1,000 times which indicates an adequate transfection efficiency. There was an increased basal gene expression for almost all mutant constructs and genes compared to the wild-type construct (Figure S4). Wnt3a-ctivated signaling resulted in significant increased AXIN2 gene expression for all *LRP5* constructs (Figure S5).

SUPPLEMENTARY FIGURES

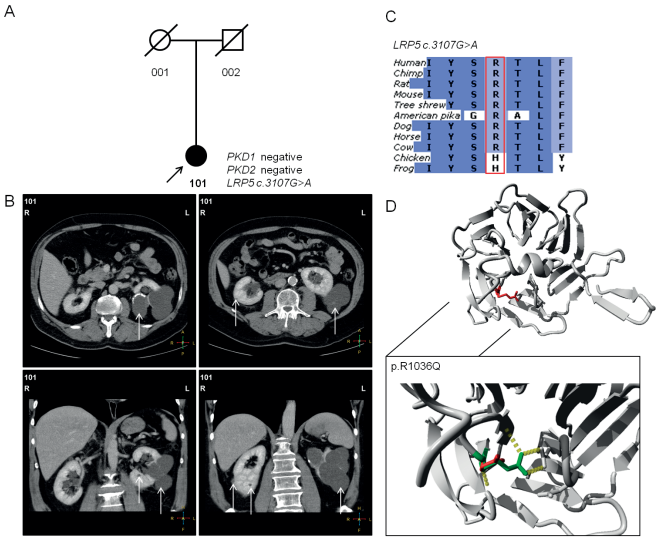

Figure S1. Family B. Pedigree of family B. (B) CT scanning of proband 101 (axial and frontal). Renal cysts are indicated by white arrows. (C) *LRP5 c.3107G>A* results in an amino acid change located in a moderate-high conserved region. (D) Homology modeling presents the consequence of the missense variant. The amino acid change of arginine results in loss of hydrogen and ion binding interactions which may destabilize the WD40 domain.

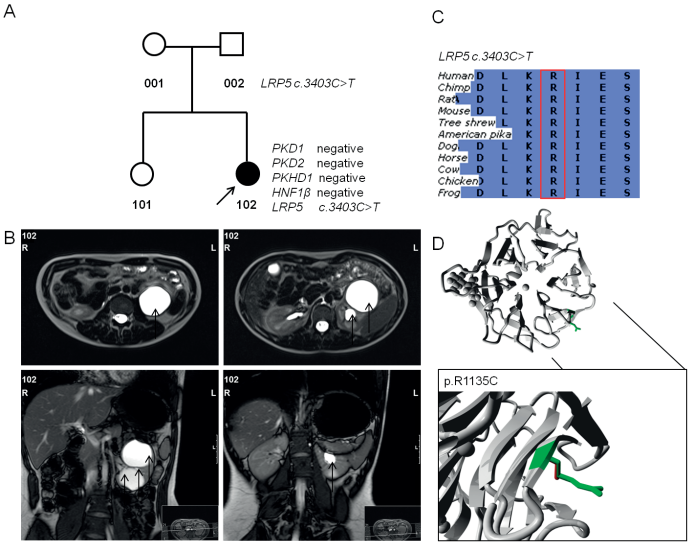

Figure S2. Family C. Pedigree of family C. (B) CT scanning presents large renal cysts in the left kidney and a small cyst in the right kidney (white arrows). (C) *LRP5 c.3403C>T* is located at an evolutionary highly conserved region. (D) Homology modeling shows that the variant results in a WD40 domain with a diminished stability.

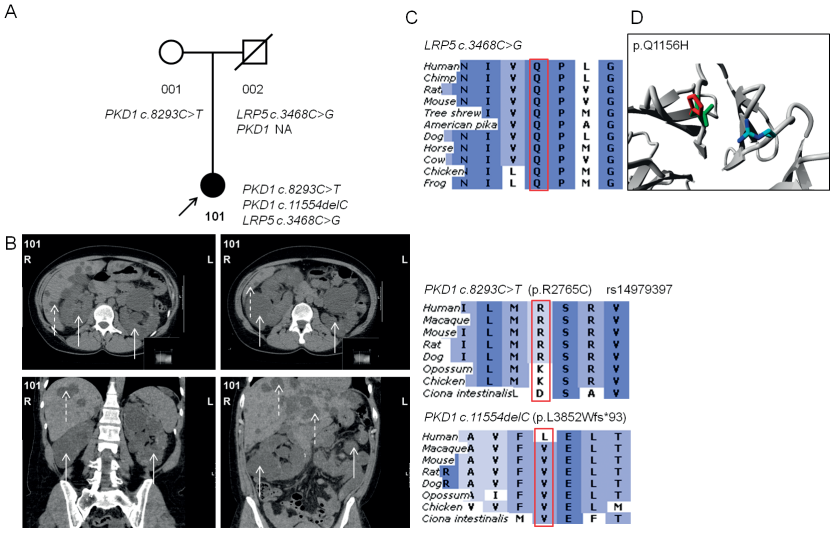

Figure S3. Family D. Pedigree of family D. (B) CT scanning before kidney transplantation presents multiple fluid-filled hepatic cysts (dotted white arrows). Polycystic kidneys are indicated by white arrows. (C) The *LRP5 c.3468C>G* is located in a highly conserved region in contract to both *PKD1* variants. (D) Change in electrostatic charge results in severely disturbed interactions at the surface of the protein domain.

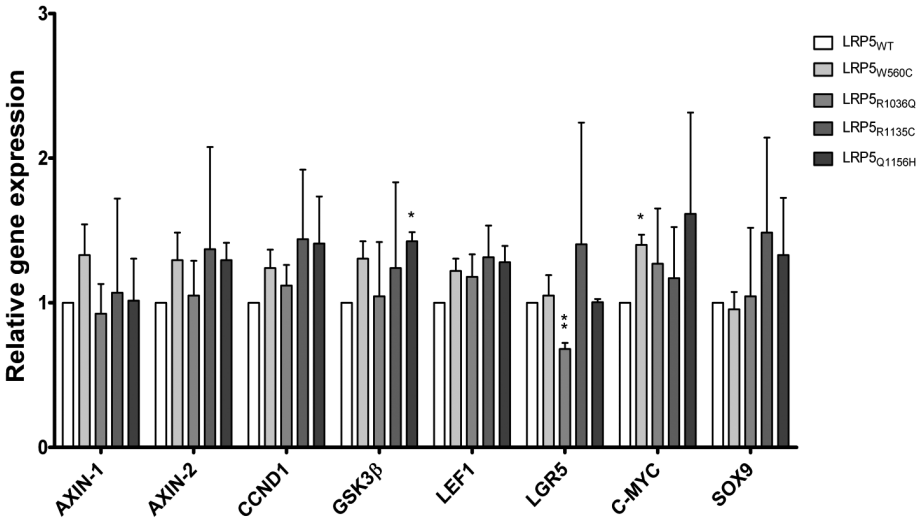

Figure S4. Basal gene expression levels. Basal gene expression levels of genes associated with the canonical Wnt signaling pathway. No Wnt3a was added to activate signaling. We corrected results for $LRP5_{WT}$ levels. The y-axis presents the relative gene expression level. One *LRP5* mutant construct showed significant *GSK3*β and *c-Myc* gene expression (*p<0.05). Significant decreased gene expression is presented in $LRP5_{R1036Q}$ (**p<0.01).

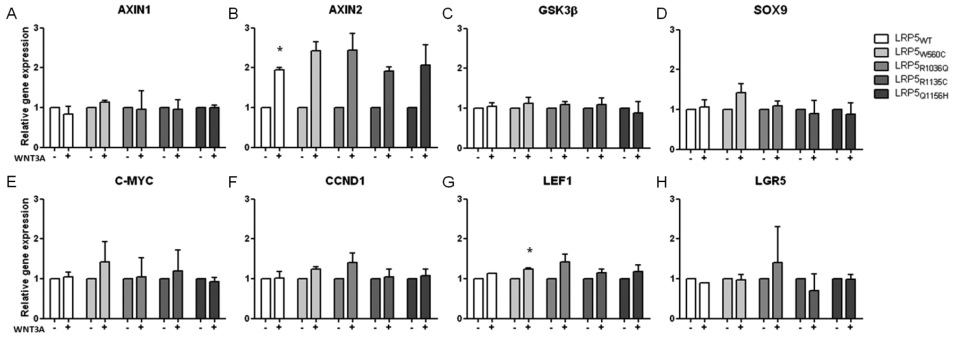

Figure S5. Gene expression levels associated with the canonical Wnt signaling pathway. Gene expression levels of (A) *AXIN1*, (B) *AXIN2*, (C) *GSK3*β involved in β-catenin degradation. Furthermore, gene expression levels of Wnt target genes *SOX9* (D) and *LGR5* (H). *C-Myc* (E), *CCND1* (F) and *LEF1* (G) function at the nuclear end point of the canonical Wnt signaling pathway. In all panels, the first bar presents the unstimulated situation (basal gene expression levels) for each *LRP5* construct. Expression levels of the second bar are the result of activation of the Wnt signaling by extracellular ligand Wnt3a. Activated gene expression levels are corrected for basal gene expression of the respective *LRP5* construct (*p<0.05). The y-axis presents the relative gene expression level.

Table S1. Clinical presentation of ADPKD index patients and family members. Index patients from families P-2, P-3 and P-4 presented a negative family history for polycystic diseases (ADPKD, ARPKD, PCLD), renal diseases or renal/ hepatic abnormalities (genomic syndromes).

Baseline characteristics				Characteristics at presentation (diagnosis)		Cystic phenotype			Clinical diagnosis	Genotype (*c.DNA*)	Treatment for polycystic kidneys
Family	Subject	Sex	Age (y)	Age (y)	Creatinin (µmol/l)	ESRD	Kidney	Liver	Polycystic or renal disease	GRCh37 (hg19)	Medical/ Surgical
A	001	F	91†	NA	NA	NA	NA	NA	NA	NA	-
	002	M	53†	NA	NA	NA	NA	NA	NA	NA	-
	109*	F	56	51	155	Y	Y	Y	ADPKD with severe PLD[ß]	*LRP5 c.1680G>T*	Long-term AHT
	202	F	33	24	79	N	Y	Y	ADPKD[ß]	*PKD1 c.1281_1283delGGC*	8-months SSA
	205	F	27	27[#]	NA	N	N	N	No ADPKD/ ARPKD/ PCLD	*PKD1 c.3133G>C*	-
	206	F	25	25[#]	NA	N	N	N	No ADPKD/ ARPKD/ PCLD	*LRP5 c.1680G>T* *PKD1 c.1281_1283delGGC* *PKD1 c.3133G>C* *LRP5 c.1680G>T* *PKD1 c.3133C*	- -
B	001	F	84†	NA	NA	NA	NA	NA	NA	NA	-
	002	M	66†	NA	NA	NA	NA	NA	NA	NA	-
	101*	M	60	55	80	N	Y	N	ADPKD[ß]	*LRP5 c.3107G>A*	Long-term AHT
C	001	F	47	44[#]		N		N	No ADPKD	*LRP5 c.3403C>T*	-
	002	M	48	45[#]		N	Y	N[α]	No ADPKD	-	-
	101	F	20	-		N		N	No screening	*LRP5 c.3403C>T*	-
	102*	F	18	14		N		N	ADPKD[ß]	*LRP5 c.3403C>T*	-
D	001	F	75	75[#]	86	N	N	N	No ADPKD; 2 adnex cysts	*PKD1 c.8293C>T*	-
	002	M	70†	70[#]	87	N	N	N	No ADPKD	*LRP5 c.3468G>C, PKD1 NA*	-
	101*	F	51	43	114	Y	Y	Y	ADKPKD[ß]	*LRP5 c.3468G>C* *PKD1 c.8293C>T* *PKD1 c.11554delC*	Long-term AHT KidneyTX

Abbreviations: P, pedigree; * Index patient; F, female; M, male; NA, not available; † Deceased and age of death; [#] age at phenotype screening by abdominal ultrasonography or most recent historical radiological imaging; ESRD, end-stage renal disease; [α] presence liver function disturbances; ADPKD, autosomal dominant polycystic kidney disease; ARPKD, autosomal recessive polycystic kidney disease; PCLD, isolated polycystic liver disease (autosomal dominant); severe PLD, refers to symptomatic polycystic liver disease with hepatomegaly; [ß] clinical diagnosis of ADPKD meets the unified Ravine criteria; AHT, anti-hypertensive treatment; SSA, somatostatin analogue (octreotide) treatment; kidneyTX, kidney transplantation.

Table S2. Genomic sequence analysis.

Family	Subject	Sex	Age (y)	Polycystic or renal disease	PKD1	PKD2	PKHD1	HNF-1ß	PRKCSH	SEC63	LRP5
A	001	F	91†	NA	-	-	-	-	-	-	-
	002	M	53†	NA	-	-	-	-	-	-	-
	109*	F	56	ADPKD with severe PLD[ß]	X	X	-	-	X	X	X
	202	F	33	ADPKD[ß]	Exon seq.	-	-	-	-	-	Exon seq.
	205	F	27	No ADPKD/ ARPKD/ PCLD	Exon seq.	-	-	-	-	-	Exon seq.
	206	F	25	No ADPKD/ ARPKD/ PCLD	Exon seq.	-	-	-	-	-	Exon seq.
B	001	F	84†	No ADPKD (meta-history)	-	-	-	-	-	-	-
	002	M	66†	No ADPKD (meta-history)	-	-	-	-	-	-	-
	101*	M	60	ADPKD[ß]	X	X	-	-	-	-	X
C	001	F	47	No ADPKD, adnex cysts	-	-	-	-	-	-	Exon seq.
	002	M	48	No ADPKD	-	-	-	-	-	-	Exon seq.
	101	F	20	Unknown	-	-	-	-	-	-	Exon seq.
	102*	F	18	ADPKD[ß]	X	X	X	X	-	-	X
D	001	F	75	No ADPKD; 2 adnex cysts	Exon seq.	-	-	-	-	-	Exon seq.
	002	M	70†[P]	No ADPKD	-	-	-	-	-	-	Exon seq.
	101*	F	51	ADKPKD[ß]	X	X	-	-	X	X	X

X, whole-gene sequence analysis; Exon seq., exon sequencing; [P], DNA derived from paraffin-embedded tissue section.

Table S3. Frequency of detected *LRP5* variants.

***LRP5* variant ; rs (family; ethnicity)**	*c.1680G>T* ; **rs377144001 (A; Moroccan)**	*c.3107G>A* ; **rs61889560 (B; Dutch)**	*c.3403C>T* ; **rs143396225 (C; Dutch)**	*c.3468G>C* ; **rs- (D; Dutch)**
NHLBI GO ESP Exome Variant Server EVS-v.0.0.22, released Oct. 17, 2013 (Allele frequency)[41]	European-American:1/8,588 (0.00012) African-American: 0/4,400 (**absent**) Overall: 1/12,988 (0.00008; **1/12,988**)	European-American: 44/8,588 (**0.0051; 1/195**) African-American: 9/4,400 (0.0020; 1/488) Overall: 53/12,988 (0.0041; **1/245**)	European-American: 7/8,588 (**0.00081; 1/1,226**) African-American: 3/4,400 (0.00068; 1/1,466) Overall: 10/12,988 (0.00077; **1/1,299**)	absent
1,000 Genomes Project, v73.37 (GRCh37), released Oct. 14, 2013[40]	absent	0.001	-	absent
GoNL[42]	absent	-0.004	absent	absent
Exome sequencing data in-house from primarily European individuals (n=2,000)[43, 44]	absent	absent	absent	absent
Conservation (GERP) (-12.3 – 6.17)[45]	4.13	3.71	4.93	-

Table S4. Predicted protein effect and frequencies of detected *PKD1* variants in family A and D.

	***PKD1* variant (*c.DNA*) (NM_001009944.2)**	**Protein effect**	**Exon**	**Protein domain**	**EVS frequency***	**Clinical significance from PKD mutation database[15]**	**Literature**
Family A	*c.1281_1283delGGC*	p.(Ala428del)	6	C-type lectin domain	NA	NA	-
Family A	*c.3133G>C*	p.(Val1045Leu)	13	5th PKD domain	NA	NA *c.3133G>A* (p.(Val1045M)); Likely hypomorphic[31]	-
Family D	*c.8293C>T*	p.(Arg2765Cys)	23	Egg jelly receptor, REJ-like	European-American: 1/67 Afro-American: 1/180 All: 1/85	Likely hypomorphic[16, 32]	32
Family D	*c.11554delC*	p.(Leu3852Trpfs*93)	42	(Polycystin cation channel, PKD1/PKD2)	NA	Definitely pathogenic [16]	16

*Genotype frequency

Table S5. *In silico* analysis of detected *PKD1* missense variants.

	***PKD1* variant (*c.DNA*) (NM_001009944.2)**	**PolyPhen2**	**SIFT**	**Align GVGD**	**PhyloP**	**Grantham score**
Family A	*c.3133G>C*	Probably damaging (0.988)	Deleterious (0.00)	C25 (GV: 0.00 - GD: 30.92)	2.87	32
Family D	*c.8293C>T*	Probably damaging (0.999)	Deleterious (0.03)	C15 (GV: 108.93 - GD: 124.09)	2.30	180

Table S6. Location of *LRP5* variants. We identified four *LRP5* variants located at extracellular LRP5 protein domains. A homology model of LRP5 protein was created using the YASARA&WHAT IF Twinset.[37] As a template, several previously solved crystal structures were used to build separate models (Table S7). For four WD40 domains (β-propeller subdomains) PDB-files were available to incorporate the identified extracellularly located *LRP5* variant.[38, 39] For the *PKD1* variant a minor domain model was available.[46] Separate models for these domains and the variants were visualized and analyzed using YASARA.

Family	Amino acid change	LRP5 domain	LRP5 location
A	p.(Trp560Cys)	2nd β-propeller; 4th blade	Extracellular
B	p.(Arg1036Q)	4th β-propeller; 3th blade	Extracellular
C	p.(Arg1135Cys)	4th β-propeller, 5th blade	Extracellular
D	p.(Gln1156His)	4th β-propeller, 5th blade	Extracellular

Table S7. Templates of homology models for LRP5 and PKD1 protein domains.

LRP5 domain	PDB ID code	LRP5 identity (%)	Reference
2nd β-propeller	3S94	79	38
4th β-propeller	4A0P	65	39
PC-1 domain	**PDB ID code**	**PC-1 identity (%)**	**Reference**
L-lectin	2XR5	29	46

Table S8. *LRP5* variants reported in this manuscript.

Family	Position (GRCh37; hg19)	Variant (*c.DNA*; NM_002335.2)	Exon	Predicted effect on protein	Known SNP	dbSNP (v.138)
A	g.68171046	c.1680G>T	8	p.(Trp560Cys)	Yes	rs377144001
B	g.68191036	c.3107G>A	14	p.(Arg1036Gln)	Yes	rs61889560
C	g.68192736	c.3403C>T	15	p.(Arg1135Cys)	Yes	rs143396225
D	g.68193486	c.3468G>C	16	p.(Gln1156His)	No	rs724159825

Table S9. *PKD1* variants reported in this manuscript.

Family	Position (GRCh37; hg19)	*PKD1* variant (*c.DNA;* NM_001009944.2)	Exon	Predicted effect on protein	Known SNP	dbSNP (v.138)
A	g.2117591	*c.1281_1283delGGC*	6	p.(Ala428del)	No	rs724159824
A	g.2112816	*c.3133G>C*	13	p.(Val1045Leu)	No	rs724159822
D	g.2153765	*c.8293C>T*	23	p.(Arg2765Cys)	Yes	rs144979397
D	g.2091581	*c.11554delC*	42	p.(Leu3852Trpfs*93)	No	rs724159823

SUPPLEMENTARY URLs

- Primer3, v.0.4.0 (latest version); http://frodo.wi.mit.edu/primer3/;[34]
- SNP Check, v.3 (latest version); a tool for performing batch checks for the presence of SNPs in predicted PCR primer binding sites; https://secure.ngrl.org.uk/SNPCheck/snpcheck.htm
- Human genome browser gateway; http://genome.ucsc.edu/cgi-bin/hgGateway; v.hg19 human reference genome (GCRh37);[35, 36]
- Polycystic Kidney Disease Mutation Database (PKDB), v.3.0: http://pkdb.mayo.edu/;[15]
- 1,000 Genomes Project, a deep catalog of Human Variation; http://www.1000genomes.org/data#DataAccess (in 1,000 individuals variants were assessed in the project);[40]
- Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA; November 2012 accessed; http://evs.gs.washington.edu/EVS/ (in 6,500 individuals variants were assessed in the project);[41]
- Database of Single Nucleotide Polymorphisms (dbSNP); http://www.ncbi.nlm.nih.gov/projects/SNP/; Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine; NCBI dbSNP Build 137; 26th June 2012 available;[47]
- Mouse Genome Informatics; v.MGI 5.17 (last database update 04-22-2014); http://www.informatics.jax.org/searches/allele_report.cgi?_Marker_key=37359; MGD[48]
- Human gene mutation database (HGMD® Professional) (www.biobase-international.com/hgmd)
- from BIOBASE Corporation; Professional 2012.4, 14th December 2012 accessed;[49]
- MRS database; v.6 (latest version); http://mrs.cmbi.ru.nl/m6/entry?db=sprot&id=lrp5_human&rq=lrp5_human;[50]
- Project HOPE, http://www.yasara.org/;[37]
- The Wnt home page, latest version June 2013: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/
- Genome of the Netherlands; GoNL; variants from ~500 Dutch individuals, release 5; http://www.nlgenome.nl/?page_id=9;[42]

CHAPTER 9

DISCUSSION AND FUTURE PERSPECTIVES

DISCUSSION AND FUTURE PERSPECTIVES

Polycystic liver disease (PLD) consists of three major adult phenotypes: Von Meyenburg complexes (VMC), isolated polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD). These separated entities may be distinguished by clinical examination and radiological imaging. All three disorders have differences in epidemiology, intra-hepatic anatomical level, symptoms and presence of extra-hepatic features to classify the phenotype accordingly. The PLD phenotype may present with a large variety of hepatic cystogenesis. The location, number and size of cysts do not directly correspond to symptoms in affected individuals.

VMC develop from dilated peripheral bile ducts into small hepatic cysts without growth advantages. In general, VMC patients remain asymptomatic. This contrasts clinical findings and patients' presentation in PCLD and ADPKD.

The clinical features and consequences of hepatic cysts trigger the **clinical diagnosis** of PCLD or ADPKD. **Symptomatic PLD** is mainly seen in these two autosomal dominantly inherited disorders. However, a proportion of asymptomatic PCLD and ADPKD patients are identified accidentally or remain unknown for hepatic cysts. Subsequently, radiological imaging such as abdominal ultrasonography is the first modality to confirm the clinical diagnosis. In order to confirm the clinical diagnosis **molecular genetic testing** is available. Identification of pathogenic mutations in the currently known genes for PCLD and ADPKD may provide valuable information for patients and clinicians.

The primary aim of this thesis was to identify the **genetic cause** in PCLD patients without a previous molecular diagnosis, in order to elucidate the pathogenesis of hepatic cysts.

In this chapter, I will first discuss the diagnostic criteria as well as the clinical phenotype assessment in probands and their family members. Next, results of the search for novel disease genes is discussed based on our identification of a novel gene associated with hepatic cystogenesis. Finally, the implications of these results are outlined to give directions for future research.

PART I POLYCYSTIC LIVER DISEASE PHENOTYPE

Diagnostic Criteria for Adult Polycystic Liver Disease

Polycystic livers are characterized by the presence of at least 20 cysts regardless of size and distribution. This practical definition is based on experience in clinical practice. Patients with severe PLD frequently visit hospitals for information and management in contrast to mildly affected individuals. Asymptomatic hepatic cysts are not extensively documented. The phenotypical expression may be different in PCLD and ADPKD and the diagnosis is primarily based on clinical features. In **chapter 2** I provide an overview of the clinical features that establish the spectrum of PLD. In general, the clinical diagnosis of both PCLD and ADPKD consists of the following three aspects: number of hepatic/ renal cysts, age of the individual and family history. In view of these criteria, I highlight several considerations:

Number of Hepatic Cysts in PCLD

Fluid-filled cysts are sharply demarcated structures on radiological imaging such as abdominal ultrasonography, magnetic resonance imaging (MRI) and computed tomography (CT). Ultrasonography is commonly used as the primary imaging modality in many diseases and also the first tool for screening individuals suspected of renal or hepatic cystogenesis.

The widespread use of ultrasonography has resulted in increased detection of cysts, and may refine the diagnosis. Abdominal ultrasonography is relatively easily diagnosis is done by counting the number of cysts of variable sizes. However, the detection sensitivity and specificity depends on the resolution of the mechanical sector probe. Regarding the diagnostic accuracy of ultrasonography a detection of hypo-echogenic cysts >1 cm is recommended.[1] Moreover, a reliable determination of the number of hepatic cysts depends on the interpretation and experience of the sonographer.

In addition, in young (<30-40-year-old) individuals with an indeterminate phenotype, other radiological imaging tools are more sensitive to detect the presence of hepatic or renal cysts. Moreover, differential diagnosis is needed for other cyst-like conditions such as echinococcal cysts, hemangioma, abscess, metastases and other hepatic malignancies.

Age of Affected Individual

Since hepatic cysts have frequently a late-onset and may increase over time, the age at diagnosis is important. Hormonal and metabolic values may change during life and this may impact the development of hepatic cysts. Subsequently, this is related to disease development and severity. Long-term follow-up of the phenotype in family members may be useful for both individual's health and insight in development of cysts during life.

Although current diagnostic guidelines take the patient's age into account, a strictly determined age cutoff for the clinical diagnosis may not always be useful. An exact age cutoffs may limit the clinical spectrum in a rare disorder with nonpenetrance.

Family History

A positive family history should be considered as a risk factor for developing autosomal dominant PLD, as offspring and siblings have 50% chance of developing cysts. Diagnosis of PLD in a patient may mandate screening for family members. During this screening process it is useful to obtain information of other liver and kidney diseases, renal failure, liver and/ or kidney transplantation, other cystic diseases, ciliopathies, and extra-renal non-hepatic features associated with ADPKD. In addition, a detailed family history comprises the origin/ lifestyle/ jobs of parents, consanguinity, causes of death in family members or autopsy information. Particular life-style factors are smoking, diet, caffeine intake, and estrogen administration (contraceptive steroids or female hormone replacement therapy). Although female steroid hormones and pregnancies are associated with hepatic cyst growth, for other environmental factors it remains unknown whether and to which degree they contribute to hepatic cystogenesis.

Current Clinical Diagnosis

In **chapter 2** the current diagnostic criteria are outlined for clinical practice. The first clinical criterion in ADPKD diagnosis consisted of bilateral renal cysts with at least two cysts in each

Table 1. Ravine and unified Ravine ultrasonography criteria for the clinical diagnosis of ADPKD.[1, 2, 6]

Ravine criteria [2]			Unified Ravine criteria [1]	
Age (y)	**Negative family history**	**Positive family history***	**Age (y)**	**Positive family history and unknown genotype**
<30	5 cysts bilaterally**	2 cysts bilaterally (or unilaterally)	15-39	≥3 cysts bilaterally (or unilaterally)
30-59	5 cysts bilaterally	≥2 cysts in each kidney (4 cysts bilaterally)	40-59	≥2 cysts in each kidney
≥60	8 cysts bilaterally	≥4 cysts in each kidney (8 cysts bilaterally)	≥60	≥4 cysts in each kidney

*Definition of a positive family history: at least one affected family member. **Bilaterally: at least both kidneys are affected.

kidney. Next, the Ravine criteria were established using 381 family members from 18 *PKD1* affected families. Clinical diagnostic criteria followed from standard ultrasonography screening criteria accompanied with DNA linkage analysis.[2] These important results provided information about the required number of renal cysts in young individuals at-risk (Table 1).

For screening families with an unknown genotype, age-dependent, unified Ravine criteria were determined by phenotyping 948 at-risk individuals from *PKD1* and *PKD2* families.[1] The most essential finding of this study was that ≥40-year-old persons with only one renal cyst ADPKD may be excluded as being patients in at-risk individuals from unlinked families. In addition, exclusion of ADPKD is supported when no renal cysts are detected in individuals with an age between 30-39 years and an unknown familial genotype.

Clear diagnostic criteria for PLCD is absent and clinical PCLD studies used different criteria;

Polycystic liver disease is characterized by the presence of >20 cysts spread throughout the liver.[3]

Table 2. Reynolds ultrasonographic criteria for the clinical diagnosis of PCLD.[5]

Reynolds criteria [5]		
Age (y)	**Negative family history**	**Positive family history***
<40	Unreported	≥1 cyst
≥40	Unreported	≥4 cysts

*Definition of a positive family history: at least 1 family member with PCLD and a clear autosomal dominant inheritance pattern.

For genotype-phenotype analysis of PCLD, patients were categorized following the number of detected hepatic cysts: (I) 1-10 cysts, (II) 11-20 cysts, and (III) >20 cysts.[4]

A group of the Mayo Clinic proposed the following age-dependent criteria based on linkage analysis study in two PCLD families (Table 2).[5] Individuals >40 years of age with one to three hepatic cyst(s) were considered as indeterminate. The reason for this definition is because of the late-onset of disease in PCLD. These are the most commonly used criteria.

Several issues question the sensitivity and specificity of current clinical diagnostic criteria in PCLD. First, both PCLD families [5] used for establishing these criteria stem from a cohort where *PRKCSH* mutations were identified.[7] This may very likely have introduced a bias in the establishment of the clinical criteria in PCLD. In addition, there is less evidence that the severity of hepatomegaly is related to the mutated gene *PRKCSH* in PCLD in contrast to ADPKD.[8] It is therefore unclear whether similar diagnostic criteria should be used for PCLD patients without *PRKCSH* mutations. In addition, there is an evidence for nonpenetrance in PCLD.[4]

High Clinical Heterogeneity in PCLD Families

For the identification of mutation-negative families 32 PCLD probands without a *PRKCSH* or *SEC63* mutation were assessed. **Chapter 3** describes clinical information in all available family members and abdominal ultrasonography performance according to the Reynolds criteria for PCLD.[5] These diagnostic criteria have several limitations as described previously, but are still the most sensible guidelines for PCLD diagnosis. Assessment of the cyst number, age and taking family history are three aspects evident in both ADPKD and PCLD diagnosis.

Although renal cystogenesis is rare in PCLD and polycystic livers are common in ADPKD, the current criteria include no statements about involvement of **cysts in other organs**. The Reynolds criteria are relevant for hepatic cysts and vice versa the Ravine criteria are relevant for assessment of renal cystogenesis.[2, 5]

When few renal cysts in a polycystic liver patient are identified, first the ADPKD criteria should be consulted. This approach is justified because up to 94% of ADPKD patients had a polycystic liver as extra-renal manifestation.[9] Secondly, evidence from *PKD1* and *PKD2* affected individuals showed that ADPKD patients are at-risk for early and progressive renal disease.[10]

In general, PCLD patients do not meet the Ravine criteria for renal cystogenesis. This contrasts ADPKD patients that meet both the Reynolds and the (unified) Ravine criteria. According to these diagnostic guidelines and clinical features in ADPKD patients, there is an important overlap between PCLD and ADPKD.

In only ~10% of ADPKD the family history was negative, but clinical characteristics of patients with sporadic and familial ADPKD were similar.[11] In line with these findings, a clinical PCLD study describes that PCLD patients without *PRKCSH* or *SEC63* mutation (~80%) present similar phenotypes with respect to symptoms compared to patients with a known PCLD mutation.[4] In chapter 3, clinical characterization of PCLD probands and family members showed large differences in disease severity and symptoms. Therefore, this study identified a significant **intrafamilial heterogeneity** in multiple PCLD families.

Clinical symptoms may be considered as the most important feature in the diagnostic work-up of PLD patients. Affected individuals may present symptoms of abdominal distension

and/or pain, dyspnea, early satiety, pyrosis and rarely abnormal liver function tests. All 32 PCLD probands showed at least one of these features described in **chapter 3**. Screening family members showed a higher incidence of hepatic cysts. From this group 5 out of 49 patients (10.2%) had symptoms of PLD. This suggests that clinical features are not indicative for PLD. The most important implication of this study is that if an individual develops abdominal symptoms, knowledge about family members with hepatic cystogenesis is important (Figure 1). In case hepatic cysts are detected by abdominal ultrasonography genetic counseling may be considered. If the genetic cause for PCLD is known in the proband, molecular genetic diagnostics may confirm the clinical diagnosis in family members. The proposed algorithm as illustrated in figure 1 is more simple and conservative in contrast to flowcharts in ADPKD.[12]

For both PCLD and ADPKD it is true that hepatic cysts are more massively enlarged in **females**.[13, 14] Pregnancies and female steroid hormones (estrogens) may contribute to hepatic cyst growth. This additional factor has no place at this moment in the diagnostic work-up and this should perhaps be changed. Also, it remains unclear whether the disease severity increases in subsequent **generations**. A prospective follow-up of patients, family members and unaffected individuals may provide more information about the extent of this influencing factor in PLD patients.

In conclusion, this study proves that the **incidence is underestimated** in PCLD by a high clinical heterogeneity (Figure 2). Clinical diagnosis of PCLD is cumbersome when hepatic and renal cystogenesis are overlapping in affected individuals (Figure 3). Taking a comprehensive family history is strongly recommended for future research and health care.[15]

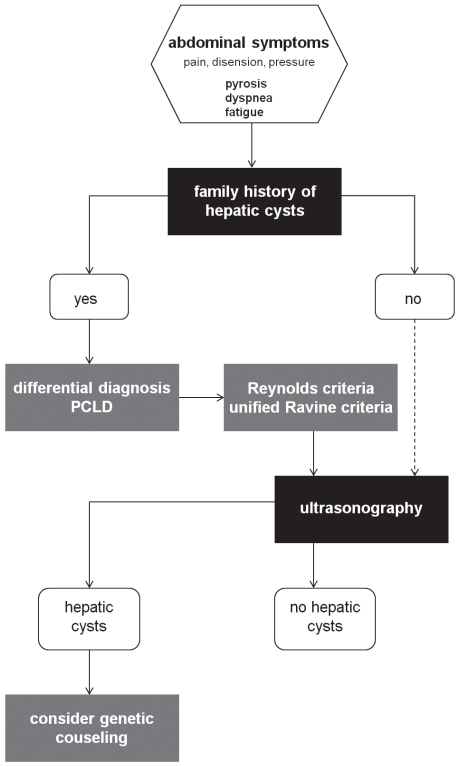

Figure 1. Diagnostic approach for individuals with PCLD-associated symptoms suspected for a polycystic liver.

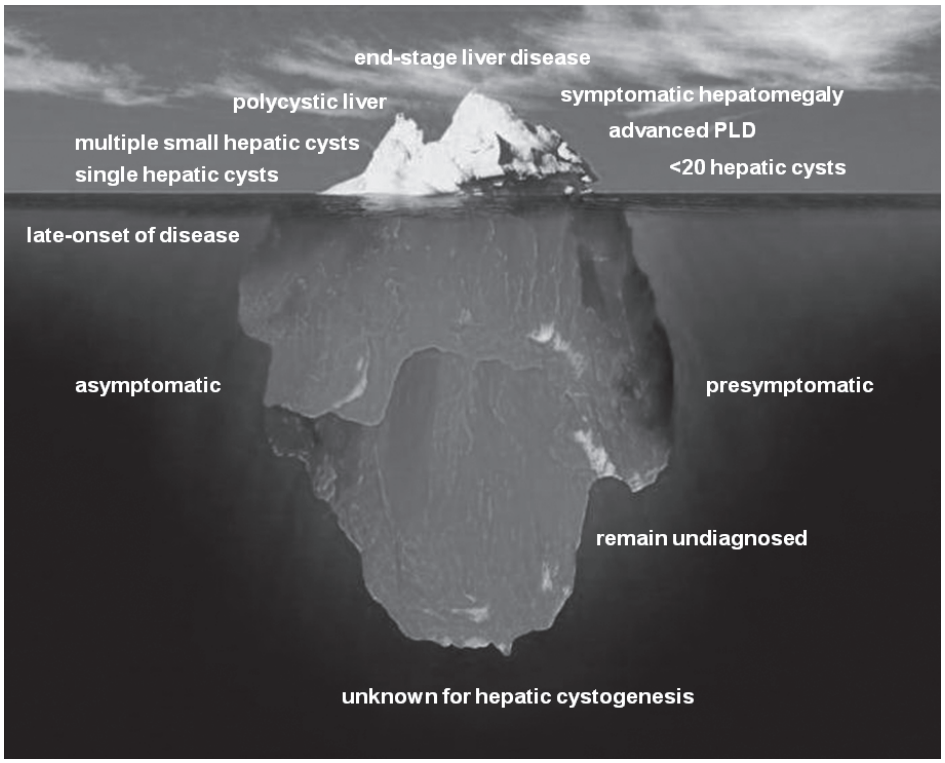

Figure 2. The spectrum of clinical presentations in hepatic cystogenesis. The large majority of patients remain unknown for hepatic cysts because of several main reasons; late-onset of disease, absence of symptoms and unrecognized presence of cysts.

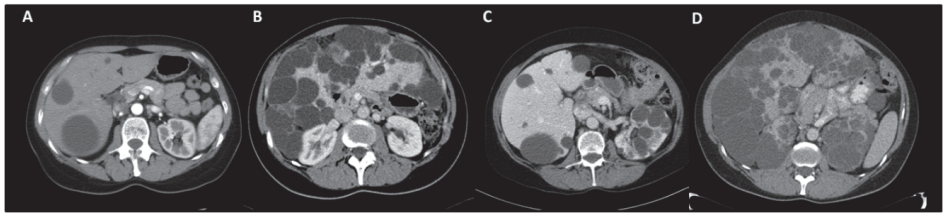

Figure 3. Clinical heterogeneity. A high clinical heterogeneity in PCLD suggests an underestimated incidence of hepatic cystogenesis. (A) PCLD patient with a mild polycystic liver. (B) Severe PCLD without involvement of renal cysts. (C) Multiple renal cysts meets the Ravine criteria, but hepatic cysts are additionally present. (D) ADPKD patient with a severe polycystic liver and renal disease meets the Ravine and Reynolds criteria.

PART II GENETIC RESEARCH FOR POLYCYSTIC LIVER DISEASE GENES

Supporting Evidence for the Search of Unknown PCLD Genes

Until now, only two genes are known to cause PCLD. *PRKCSH* has been suggested as the most important gene for PCLD because of its high mutation frequency.[4, 16] Ever since the identification of the *PRKCSH* and *SEC63* gene in 2003 and 2004, no additional PCLD gene has been described, but also no comprehensive genetic studies were performed in this field.[7, 17, 18]

I hypothesized that mutation-negative PCLD patients may be explained by CNVs in the *PRKCSH* gene. Large pathogenic CNVs are not detected by conventional Sanger sequencing which is routinely used for PCLD diagnosis.

I performed MLPA assays in DNA derived from hepatic cyst epithelium, but identified no exon deletions or duplications. This was probably due to an originally heterogeneous cell population (or mosaicism) and secondly, DNA was amplified from small numbers of cells which may reduce CNV detection sensitivity. In conclusion, no pathogenic deletions of the *PRKCSH* gene on genomic and somatic level were identified. These findings indicate that MLPA analysis of the *PRKCSH* gene has no additional value for inclusion in the diagnostic work-up of PCLD. The question remains whether or not PCLD patients harbor CNVs affecting other genes.

The results in **chapter 4** suggest that pathogenic deletions and duplications are generally rare in genomic DNA from PLD patients. However, detailed large scale CNV screen have not routinely been performed. In ADPKD, up to 4% of patients from the CRISP cohort showed genomic rearrangements.[19] This included cases with extensive deletions affecting both the *TSC2* and *PKD1* gene (*PKD1/TSC2* contiguous gene deletion syndrome).[19] In addition, deletions in the *PRKCSH* gene on the somatic level were identified previously.[20] Based on this work in ADPKD, further research into the role of CNVs in PCLD patients is also warranted.

Genomic and Somatic Loss of Alleles Identifies Candidate PCLD Genes

The **second hit hypothesis** in polycystic liver disease was first proposed in renal cystic tissues from ADPKD patients.[21, 22] Homozygous inactivation of PLD genes at the cellular level by a second hit mechanism has been implicated in hepatic cyst formation. The purpose of **chapter 5** was to outline a synopsis of second hits in polycystic liver tissues derived from ADPKD and PCLD patients.

In ADPKD patients only a subset of hepatic cysts showed somatic hits. Identification of somatic mutations and deletions in hepatic cyst epithelium cells was determined by microsatellite studies and fragment analysis.[22-24] Tissue specific homozygous regions were identified by amplification and comparison of cyst-derived DNA and control DNA derived from blood.

Recently, somatic hits have also been identified in PCLD liver tissue, resulting in the inactivation of the second *PRKCSH* allele. These results confirmed the cellular recessive mechanism for cyst formation.[20, 25] Evidence for a digenic inheritance model was not obtained after screening the *PKD2* and *SEC63* genes in hepatic cyst DNA.[26]

This molecular mechanism of loss of heterozygosity (LOH) was used to our advantage: LOH regions can directly point to the **location of the germline mutation** on the other allele. A novel approach for a large-scale identification of unknown PCLD genes was designed based

on this observation. In **chapter 6** hepatic cyst fluid derived from aspiration sclerotherapy was collected. Hepatic cyst fluid was used to retrieve DNA derived from hepatic cyst epithelium cells. Normally, the cyst fluid is a waste after the aspiration procedure. In **chapter 6** I describe the identification of large regions of somatic LOH in 2 out of 8 cases and a large CNV. These preliminary data may point us to novel PCLD genes. As an alternative disease gene identification strategy, direct screening of somatic events in hepatic cyst DNA from ADPKD and PCLD patients with exome or genome sequencing approaches may reveal novel disease genes.[27]

The Connection between the *LRP5* Gene and PCLD

Identification of the genetic cause in an extended family with a clear autosomal dominant inheritance pattern of PCLD is outlined in **chapter 7**. For a rare disorder, this large family is very unique. Since the traditional approach of linkage analysis hampered significant progress of disease identification, whole-exome sequencing revealed the ***LRP5* gene causing hepatic cystogenesis**.

Clinical Heterogeneity

The 40-member family presented with a spectrum of severe and mild PCLD largely fulfilling the Reynolds criteria.[5] Abdominal distension and mechanical symptoms were also present in six family members with severe PCLD, but these affected individuals were not referred for management.

Abdominal ultrasonography detected in 16 individuals a PCLD phenotype, but 22 individuals carried *LRP5 c.3562C>T* (p.R1188W). This discrepancy is probably explained by late-onset of disease in three family members <40-years-old. Two males with the age of 50 and 78 years presented few renal cyst not meeting the (unified) Ravine criteria. In addition, another 49-year-old male is an example of incomplete penetrance. Since all three individuals that showed no symptoms (yet) are males, the reduced phenotype expression may also be related to the gender. This contrasts the findings in a 51-year-old male with severe polycystic liver and polycystic kidneys. His clinical features meet the Ravine criteria. This affected member was under treatment for hypertension, but had no abdominal symptoms.

Functional Analysis

The primary aim was to investigate the consequences of the identified *LRP5* variants on the signaling cascade and to compare previous functional analyses of *LRP5* variants. The functional effects of other pathogenic *LRP5* variants in eye and bone disorder have been investigated previously (Table 3). For familial exsudative vitreoretinopathy (FEVR) as well as osteoporosis pseudoglioma (OPPG) *in vitro* analyses in different cell line systems were performed to determine their effect on the Wnt signaling activity. For example, monkey fibroblast COS-7 cell line [28] and the mouse fibroblast cell line NIH3T3 have been applied for the study of OPPG [29], and the HEK293 cell line were used to establish the mechanism in FEVR.[30] Preferably, the consequences of *LRP5* variants have to be studied in the tissue of origin.

*Pathogenic LRP5 Mutations in 8 Allelic Diseases (MIM*603506)*

An important question is why multiple phenotypes are caused by variation in the *LRP5* gene. There is no straightforward answer to this question and I consider several possible options

Table 3. Reports and results of luciferase activity assays of nascent *LRP5* variants from different disorders.

c.DNA LRP5 mutation	Protein change	Functional effect on Wnt signaling	Disease [Reference]
Protein domain: 3rd blade of the 2nd β-propeller domain			
c.1300G>A	p.Asp434Asn	*Dual luciferase assay (HEK293 cells expressing WT):* Measuring firefly luciferase activity from Topflash reporter <50% Wnt1 or Wnt10b induced activity compared to *LRP5*-WT Subsequent addition of *LRP5*-WT restored activity	OPPG [30]
c.1321G>A	p.Glu441Lys	Evolutionary highly conserved position Change to lysine is likely to disturb normal protein function	FEVR [31]
c.1330C>T	p.Arg444Cys	Evolutionary highly conserved position Predicted outcome of amino acid change is loss of activity	FEVR [32]
c.1360G>A	p.Val454Met	*Dual-Glo luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Significant increased Wnt3a induced Wnt signaling activity compared to *LRP5*-WT	PCLD
c.1364C>T	p.Ser455Leu	Dual luciferase reporter assay (HEK293T cells): Significantly 40% reduced Wnt1 induced signaling activity compared to *LRP5*-WT Evolutionary highly conserved position	Idiopathic osteoporosis [33]
c.1378G>A	p.Glu460Lys	No functional analysis	OPPG [30]
Protein domain: 4th blade of the 2nd β-propeller domain			
c.1680G>T	p.Trp560Cys	*Dual-Glo Luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Significant decreased Wnt3a induced Wnt signaling activity compared to *LRP5*-WT	**ADPKD** [chapter 8]
c.1709G>A	p.Arg570Gln	No functional analysis *Dual luciferase assay (HEK293 cells expressing WT):* <50% Wnt1 or Wnt10b induced activity	AR FEVR [34] OPPG [30]
c.1708C>T	p.Arg570Trp	*Dual luciferase assay (COS-7):* Decreased Wnt3a induced Wnt signaling activity of *LRP5ΔC* and *LRP5ΔTM* compared to *LRP5*-WT	OPPG [28]
c.1750C>T	p.Gln584Term	No functional analysis	OPPG [30]
Protein domain: 3rd blade of the 4th β-propeller domain			
c.3107G>A	p.Arg1036Gln	*Dual-Glo luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Significant decreased Wnt3a induced Wnt signaling activity compared to *LRP5*-WT	**ADPKD** [chapter 8]
c.3107G>A	p.Arg1036Gln	Evolutionary highly conserved amino acid position No functional analysis *Luciferase gene reporter assay (CHO cells with Wnt3a-conditioned medium):* ***24% reduced Wnt signaling activity*** compared to the control	Primary osteoporosis [35] [36]

Table 3. ***Continued.***

***c.DNA LRP5* mutation**	**Protein change**	**Functional effect on Wnt signaling**	**Disease [Reference]**
c.3232C>T	p.Arg1078Term	No functional analysis	OPPG [30]
c.3295G>T	p.Asp1099Tyr; (4th blade)	No functional analysis	OPPG [30]
Protein domain: 5th blade of the 4th β-propeller domain			
c.3403C>T	p.Arg1135Cys	*Dual-Glo luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Decreased Wnt3a induced Wnt activity compared to *LRP5*-WT	**ADPKD** [chapter 8]
c.3468G>C	p.Gln1156His	*Dual-Glo Luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Significant decreased Wnt3a induced Wnt signaling activity compared to *LRP5*-WT	**ADPKD** [chapter 8]
Protein domain: 6th blade of the 4th β-propeller domain (AA 1165-1207)			
c.3502T>C	p.Tyr1168His	*In silico analysis:* Highly conserved residues *Homology modeling:* Mutations in the core of the protein are likely to cause destabilization of the protein fold *Dual-Glo Luciferase reporter assay (HEK293T cells):* ***Decreased Wnt1 induced Wnt signaling activity compared to LRP5-WT*** Increased Wnt1+ extra *LRP5* WT induced Wnt signaling activity compared to Wnt1 induced Wnt signaling activity (=restoration of the activity) *Dual luciferase assay (HEK293 cells expressing WT):* Addition of Wnt1 or Wnt10b showed no activity	FEVR [37] FEVR [30] OPPG [30]
c.3553G>A	p.Gly1185Arg	*Super TOP Flash reporter assay (CHO cells):* Wnt3a-CM medium increased the Wnt signaling activity compared to corresponding constructs with 10% FBS-DMEM ***No Wnt signaling activity change*** of Wnt3a-CM compared to Wnt3a-CM *LRP5*-WT	Autosomal dominant primary osteoporosis [36]
c.3562C>T	p.Arg1188Trp	*Dual-Glo Luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Significant decreased Wnt3a induced Wnt signaling activity compared to *LRP5*-WT (30-45%)	**PCLD** [38]
Intracellular protein domain: Between PPPSP motif A – B			
c.4574C>T	p.Ala1525Val	*Topflash luciferase reporter assay (C3H10T1/2 cells):* [39] 70% increased LRP5 signaling compared to *LRP5*-WT (intracellular constructs) Normal proliferation, altered osteoblastic differentiation	FEVR [37] Variable BMD [40]
c.4587G>C	p.Arg1529Ser	*Dual-Glo Luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Significant increased Wnt3a induced Wnt signaling activity compared to *LRP5*-WT	**PCLD**

Table 3. ***Continued*****.**

***c.DNA LRP5* mutation**	**Protein change**	**Functional effect on Wnt signaling**	**Disease [Reference]**
c.4600C>T	p.Arg1534Term	No functional analysis	OPPG [30]
c.4609G>A	p.Ala1537Thr	*Luciferase activity assay (HEK293T cells):* No altered Wnt1 induced Wnt signaling activity compared to *LRP5*-WT	Idiopathic osteoporosis [33]
Intracellular protein domain: Between PPPSP motif B – C (AA 1546-1573)			
c.4643G>T	p.Cys1548Phe	*Super TOP Flash reporter assay (STF cells):* ***29% reduced Wnt signaling activity*** compared to the control	FEVR [41]
c.4651G>A	p.Asp1551Asn	*Dual-Glo Luciferase reporter assay (CHO cells):* Increased Wnt signaling activity compared to the empty vector Significant decreased Wnt3a induced Wnt signaling activity compared to *LRP5*-WT (30-45%)	**PCLD** [38]

that help to understand why the identified *LRP5* mutations are linked to a liver phenotype. These arguments are based on published data, specific *LRP5* gene-related characteristics and examples from common genetic mechanisms in affected tissues.

Osteoporosis pseudoglioma (OPPG) syndrome is the first disease that was linked to *LRP5* mutations.[28] This autosomal recessive disease is characterized by a very low bone mass and ocular pathology. The authors reported that obligate carriers (parents of OPPG patients) have only a reduced bone mass, but they went further to state that they did not perform a **systematic search** for eye abnormalities in OPPG patients and their family members.[28]

The same is true for other clinical investigations of the human body. In general, patients **report symptoms**, but other malformations remain unknown during life until these develop into symptomatic disease. Affected PCLD individuals may also remain asymptomatic until late adulthood. I investigated the 40-member PCLD family for any bone or eye disorders and was able to exclude these. Vice versa, individuals affected with familial exsudative vitreoretinopathy (FEVR) or osteoporosis associated with *LRP5* variants are not **systematically** screened for liver or kidney disease (cystogenesis).

A previous study hypothesized that the pathogenesis of visual loss in OPPG is distinct from that observed in FEVR and neovascular inflammatory retinopathy (VRNI).[28] It is therefore reasonable to speculate that *LRP5* mutations could cause a spectrum of eye pathologies through **different mechanisms of mutational effect**.[28, 42] For example, dosage effects are proposed to be common in *LRP5* because heterozygous *LRP5* variants cause reduced bone mass in OPPG patients.[28]

How *LRP5* mutations specifically give rise to developmental onset of blindness or FEVR and osteoporosis in OPPG patients is unresolved.[30] Studies in *Lrp5*$^{-/-}$ mouse models indicate that the LRP5 protein uses **different molecular mechanisms** to regulate bone formation and eye vascularization.[43] Defective LRP5-signaling affects retinal vasculature through reduced level of capillary cell apoptosis and a defect in hyaloid vessel regression, resulting in a lower number of

vessel branches.[43, 44] One of the explanations is that the LRP5 signal transduction pathway, and downstream Wnt proteins, regulate both osteoblast proliferation, function, and eye vascularization.

An example is a *Lrp6*$^{-/+}$ mouse model with a hypomorphic *Wnt3a* allele presenting a more severe phenotype in contrast to presence of a normal *Wnt3a* allele.[45]

Phenotypic Expression

Further, the **incomplete penetrance** (10-33%) of the ocular phenotype and high **intra-familial heterogeneity** in FEVR families indicate that the *LRP5* genotype-phenotype is not as straightforward as it would seem.[46] To date, it remains unsolved why several family members with a bone disorder additionally develop FEVR.[30]

LRP5 gene mutations are apparently associated with both extremes of bone density. Autosomal dominant high-bone-mass disease (HBM) was mapped to the *LRP5* gene by two groups.[29, 47] These authors identified clinical differences in this low density bone disease in affected family members and between non-related patients. Remarkably, one missense mutation (p.G171V) was present in both non-syndromic and syndromic HBM. This suggests that **environmental factors** may influence the expression of phenotypic manifestations.[29,

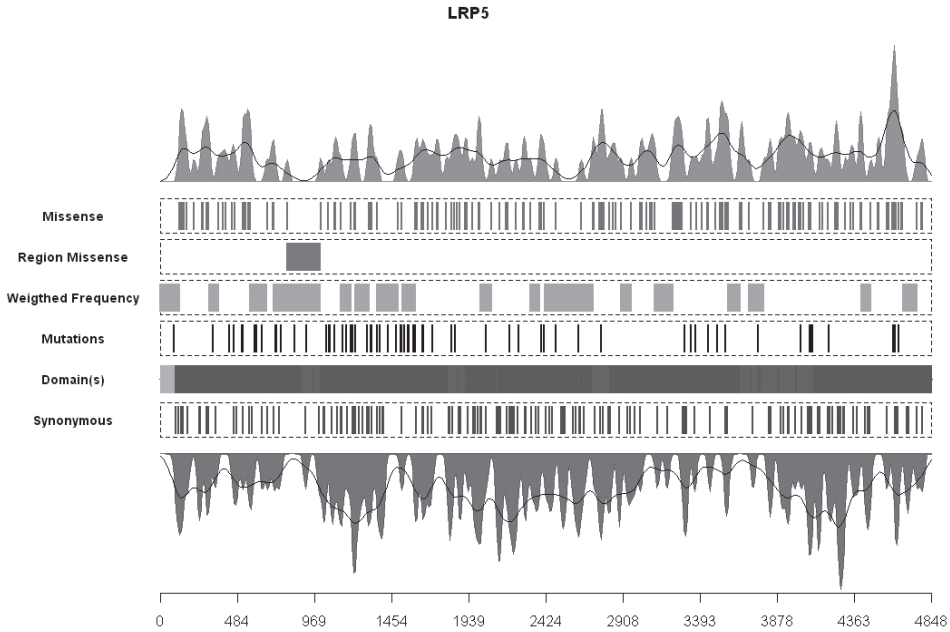

Figure 4. An overview of the *LRP5* gene (NM_002335.2; 1,615 amino acids). On the x-axis 4,848 protein-coding positions are depicted (c.DNA). Six rows present the characteristics of normal *LRP5* gene variation in 13,000 exomes (based on 6,500 EVS exomes). The frequency of missense and synonymous variants are illustrated by height of two plots. The rows in between represent the density of missense variants, a region without significant missense variation by stringent analysis, weighted frequency boxes are regions with less variants than predicted, *LRP5* mutations (HGMD) and LRP5 protein domains (Uniprot). This figure shows a clear locus heterogeneity for mutations. Although there are still regions without variation, the majority of mutations are extracellularly located. Figure 4 is kindly provided by dr. C. Gilissen.

[48] For example, *LRP5* inactivation impairs postnatal bone formation by enhancing osteoblast-directed bone resorption depending on the extracellular mediator serotonin in the gut.[49]

In addition, the (various) effects of *LRP5* mutations in bone diseases and FEVR might depend on the presence or absence of other **genetic variants**. In this respect, it is reasonable to speculate that genetic variation in other genes of the Wnt signaling pathway contribute to different phenotypes.[34, 48]

LRP5 Gene-related Characteristics

The LRP5 protein has a **wide tissue expression** such as in heart, liver, kidney, pancreas, spleen, prostate, ovary, small intestine and colon has been determined by northern blotting and in situ hybridization studies.[42, 50] All known *LRP5* variants for different phenotypes are spread over all **protein domains** and are not restricted to the extracellular part of the protein (Figure 4). Only for high bone mass disease there is a hot-spot to the first β-propellor domain.

Missense *LRP5* mutations may cause a variety of clinical presentations and have been found in individuals with OPPG, autosomal dominant and autosomal recessive FEVR. It needs to be determined whether *LRP5* mutations result in a protein loss-of-function or gain-of-function of LRP5. From *LRP5* knock-out mouse studies it is known that a variety of tissues can be affected and lead to a low bone mass, hyperlipidemia, hypercholesterolemia and an impaired glucose tolerance.[45, 51, 52] **Chapter 7** describes the gene expression results and staining experiments. Abundant LRP5 and β-catenin staining in hepatic cyst tissue sections showed that the proteins are present in this tissue. This may suggest that the underlying defect is a reduced LRP5 function due to abnormal protein structure.

Other Examples of Allelic Diseases

Altogether, *LRP5* seem to cause a spectrum of allelic disorders. This is not the first gene for which allelic heterogeneity exists. Other examples include *p63* and *PTPN11*. Gene mutations in the *p53* gene are associated with seven allelic diseases (five syndromic and two non-syndromic).[53] Missense mutations in the *p63* gene lead to very different phenotypes such as ectodermal dysplasia and cleft lip/palate syndrome. The mutations seen in these disorders may result in either a gain-of-function or loss-of-function. This is consistent with significant **genotype-phenotype variability**.

Similarly, missense mutations in the *PTPN11* gene leads to spectrum of diseases. Gain-of-function mutations may cause Noonan syndrome or juvenile myelomonocytic leukemia.[54, 55] The Leopard syndrome is an autosomal dominant disorder caused by *PTPN11* gene mutations and is characterized by lentigines and cafe-au-lait spots, facial anomalies, and cardiac defects.

ADPKD and *LRP5* Variants

Polycystic liver disease is the most common extra-renal manifestation in ADPKD patients. The prevalence of hepatic cystogenesis in ADPKD increases by age. Hepatic cysts are present in 94% of ADPKD patients older than 35 years.[9] As a logical extension of previous findings, all 23 exons of the *LRP5* gene patients were sequenced in 79 ADPKD patients. This cohort consisted of ADPKD probands without a pathogenic variant on either *PKD1* or *PKD2* (n=29), and patients

with adult-onset disease with an apparently negative family history (n=50). In the latter group no mutation analysis of ADPKD genes were performed. **Chapter 8** presents segregation analysis in several cases and functional analysis in order to identify the consequences of these *LRP5* variants. In total 4 rare *LRP5* variants were identified that led to amino acid change.

To our knowledge these are the first ADPKD patient with identified *LRP5* variants. The genotype-phenotype correlation however is not yet fully understood, but argues against a simple monogenic role of *LRP5* in ADPKD.

FUTURE PERSPECTIVES

Improvements of high-throughput sequencing technologies has shifted the laboratory work. Faster tools replaced conventional methods. Although these techniques are available, the use of the correct model for evaluating the clinical relevant hypotheses (or questions) should be critically considered.

Deep Phenotyping

Since sequencing technologies are developing rapidly, the work of the clinician should be to improve interpretation of the numerous variants detected. Detection of variants in novel genes requires validation in large cohorts, but importantly also in family members. A complete family history and follow-up, especially in first-degree related individuals is strongly recommended. This information is essential for the search of novel (modifier) genes and genotype-phenotype relations.

Next-Generation Sequencing

Whole-exome sequencing (WES) is at present the most important tool to identify disease genes. The study design in which WES is applied depends on the observed inheritance pattern. For autosomal dominantly inherited disorders large families are needed for a linkage based strategy. PCLD is a rare disease and such large families are infrequent. To bypass this issue there are more approaches available for disease gene identification.

Exome sequencing in combination with a candidate gene approach: Candidate genes identified by exome sequencing in a small cohort of patients are screened by molecular inversion probes in very large cohorts of cases and controls.[56]

Overlap based strategy: Exome sequencing of many single cases with a particular subtype of PCLD.[57]

Siblings strategy: Exome sequencing of affected siblings.

Trio strategy: Exome sequencing of unaffected parents and affected offspring when a *de novo* mutation is suspected.

Of interest in this aspect, a genetic interaction network of PLD genes has been proposed.[58] This may argue for a candidate gene approach for PCLD genes with association of proteins in the canonical Wnt signaling, ciliary genes, animal models with hepatic and/or renal cystogenesis and proteins in the endoplasmic reticulum. A question, however, is how complete such a candidate gene approach is. Unbiased exome or even genome sequencing will be essential to complete the list of PLD genes and these approaches are becoming more and more affordable.

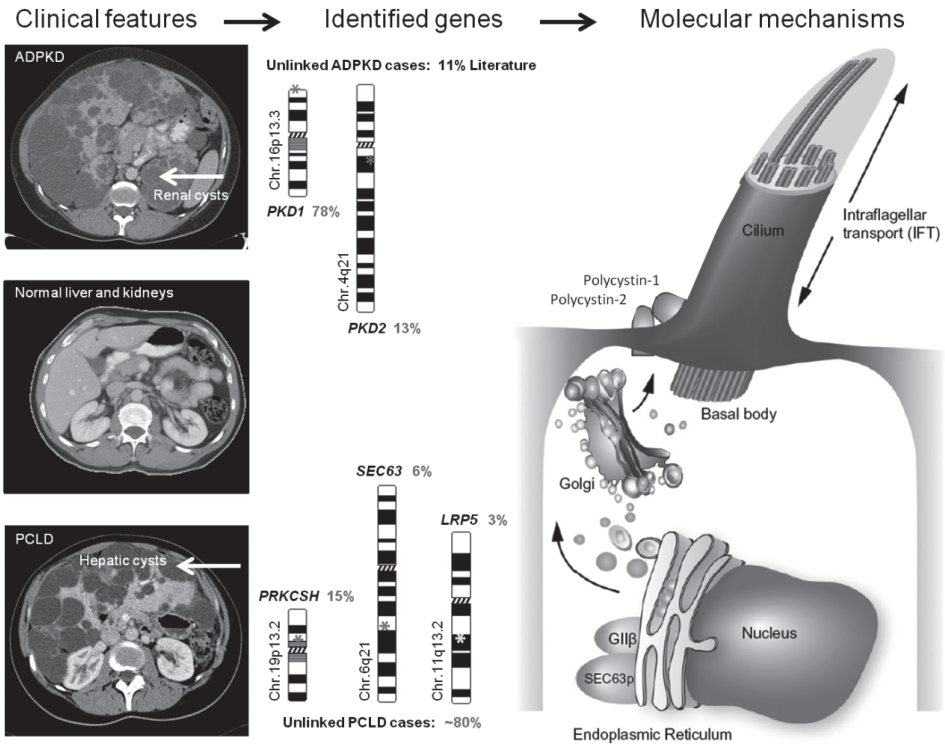

Figure 5. From patient to gene and molecular mechanism. In the opposite direction possible medical regiments may be tested for PLD management (figure partially derived from Bergmann *et al.*[60]).

Functional Studies in LRP5

The identification of *LRP5* is just the starting point for functional studies that may also result in the identification of more key players in the canonical and non-canonical Wnt signaling. The latter is associated with ADPKD and an imbalance between both may trigger cystogenesis.[59] These functional studies may include protein-protein interaction studies, (b) *LRP5* knock-down studies in human cell lines, as well as (c) *LRP5* known-down studies in animal models such as zebrafish.

In conclusion, the identification of the *LRP5* gene sets another view on PCLD and cystogenesis in general. The role of LRP5 with a connection between the endoplasmatic reticulum and cilia is of great interest (Figure 5). Functional analysis in disease models may provide more clues to the dysregulated Wnt signaling pathway and currently studied pathways for treatment. Further research should be focused on the three aspects as described above; deep phenotyping, genetic research and functional studies. It is essential to move from the clinical studies to the molecular mechanisms and back to the patient, in order to correlate clinical and molecular findings in this field. This translational research may have major impact for management of cystogenesis in patients.

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CHAPTER 10

SUMMARY - SAMENVATTING

SUMMARY

Polycystic liver disease (PLD) is a genetic disease characterized by multiple fluid-filled cysts spread throughout the liver. Patients may have an enlarged liver, but frequently a preserved liver function. Hepatic cysts may increase in number and size resulting in mechanical symptoms such as abdominal pain and pressure on surrounding organs (pyrosis and dyspnea). Complications mainly occur due to massive hepatomegaly.

PLD is a clinical diagnosis and primarily assessed by abdominal ultrasonography. Genetic molecular testing may confirm the clinical diagnosis. Three major disorders are characterized by multiple hepatic cysts: Von Meyenburg complexes, isolated polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD) (**chapter 2**). Both PCLD and ADPKD are inherited in an autosomal dominant fashion which states that 50% off the offspring also carry the mutation (DNA sequence alteration). Almost all ADPKD patients harbor a mutation in the *PKD1* or *PKD2* gene (protein-coding DNA). In PCLD, about ~80% carry no mutation in the *PRKCSH* or *SEC63* gene. The cause of hepatic cystogenesis remains unknown in the large majority of PCLD patients. Therefore, the aim of this thesis is to reveal the genetic cause in these patients.

Chapter 3 sets out how PCLD patients with unknown genomic cause often report a negative family history of liver and renal (cystic) disease. Screening family members presented frequently asymptomatic individuals and a high intrafamilial clinical heterogeneity for hepatic cystogenesis. This demonstrated an underestimated incidence of hepatic cystogenesis.

Mutation analysis consists of Sanger sequencing to detect point mutations and small deletions or duplications. **Chapter 4** examined large deletions and duplications of the *PRKCSH* gene in germline DNA. Multiplex ligation probe-dependent amplification assays were performed, but identified no copy number variations of the *PRKCSH* gene. These results argue for involvement of one or more novel genes in PCLD.

Among these PCLD patients and families of unknown genetic cause, I analyzed the DNA by using several approaches. The mechanisms of somatic inactivation in ADPKD and PCLD are discussed in **chapter 5**. Besides a first hit, PCLD patients frequently harbor a second event in liver tissue responsible for hepatic cystogenesis. This information, and the fact that a minority of PCLD patients carry a mutation in the *PRKCSH* (15%) or *SEC63* (5.7%) gene, triggered the studies described in the following chapters of the thesis. Furthermore, we searched for large homozygous regions in DNA of cyst-lining cholangiocytes derived from hepatic cyst fluid that occurred as somatic events. **Chapter 6** reports a candidate gene list (first hit) associated with PCLD.

In **chapter 7** we identified an extended PCLD family and performed whole-exome sequencing in two affected individuals. Our study presented that the only private non-synonymous variant that segregated completely with the disease is a missense mutation located on chromosome 11q13.2 in the *low-density lipoprotein receptor-related protein 5* (*LRP5*) gene. Screening of a PCLD cohort revealed 3 additional *LRP5* mutations in unrelated PCLD families (2.7%). Histology studies showed that the LRP5 protein was abundantly present in hepatic cyst tissue. In addition, functional analyses demonstrated the protein to be less functional because of a reduced activated canonical Wnt signaling pathway.

Subsequently, we investigated the role of the *LRP5* gene in ADPKD patients in **chapter 8**. Direct Sanger sequencing showed one novel and three previously reported *LRP5* variants. In addition, we reported three novel and one known *PKD1* variants. All *LRP5* variants are located on highly conservative domains and three variants are assessed to be pathogenic. Functional studies presented a decreased signal activation of canonical Wnt signaling. Gene variants may change the protein product functionally or structurally, but may also impact other interacting genes and proteins in the network. In the context of *LRP5* variants, a defective Wnt signaling may lead to an imbalance between canonical and non-canonical signaling events resulting in hepatic and/or renal cystogenesis.

In conclusion, my thesis shows that phenotyping is important in PCLD. Secondly, the genetic heterogeneity is even broader than expected. Finally, the *LRP5* gene is a new PCLD gene.

SAMENVATTING

Polycysteuze leverziekte (PLD) is een genetische aandoening waarbij meerdere met vocht gevulde cysten verdeeld in de lever aanwezig zijn. Patiënten hebben vaak een vergrote lever, maar de leverfunctie blijft intact. Levercysten kunnen in aantal en grootte toenemen met mechanische klachten als gevolg, zoals buikpijn en druk op omliggende organen (maagklachten en kortademigheid). Sterke toename van de levergrootte kan leiden tot ernstige complicaties.

PLD is een klinische diagnose en wordt dikwijls met een echo van de buik vastgesteld. Genetisch onderzoek kan de diagnose bevestigen. Met name drie aandoeningen zijn bekend met het ontwikkelen van vele levercysten: Von Meyenburg complexen in de lever, geïsoleerde polycysteuze leverziekte (PCLD) en autosomaal dominante polycysteuze nierziekte (ADPKD) (**hoofdstuk 2**). Voor PCLD en ADPKD geldt dat beide ziekten autosomaal dominant worden overgeërfd. Dit betekent dat één ouder een mutatie (afwijking) heeft in het *PRKCSH* gen of *SEC63* gen (deel van het DNA coderend voor een eiwit). De kans om de ziekte PCLD door te geven aan een kind is dan 50%. Bij ADPKD patiënten is er een mutatie in het *PKD1* of *PKD2* gen aanwezig die de ziekte veroorzaakt. Bijna alle ADPKD patiënten ontwikkelen de ziekte door een mutatie in het *PKD1* of *PKD2* gen. Voor PCLD geldt dat er geen mutatie is gevonden in het *PRKCSH* of *SEC63* gen bij ongeveer ~80% van de patiënten. De oorzaak van levercysten bij deze grote groep is onbekend. Het doel van dit proefschrift is om de genetische oorzaak te achterhalen bij deze patiënten.

Hoofdstuk 3 beschrijft hoe PCLD patiënten met een onbekende genetische oorzaak vaak geen familieleden met een polycysteuze lever- of nierziekte hebben. Bij screening met een buikecho worden alsnog cysten gevonden bij familieleden zonder klachten. Dit toont aan dat het ziektebeeld heterogeen is binnen families. Eveneens wordt de incidentie van levercysten onderschat.

Mutatie analyse (genetisch onderzoek) wordt uitgevoerd met behulp van de Sanger sequencing techniek om eenvoudige mutaties, zoals puntmutaties, deleties of duplicaties op te sporen. In **hoofdstuk 4** is onderzoek gedaan naar grote DNA veranderingen van het *PRKCSH* gen met behulp van multiplex ligation probe-dependent amplification assays. Echter, er werden geen grote deleties of duplicaties gevonden in het *PRKCSH* gen. Dit leidde tot de vraagstelling of een mutatie op één of meerdere andere genen verantwoordelijk is voor het ontwikkelen van PCLD.

Met behulp van verschillende benaderingen werd het DNA van PCLD patiënten en familieleden onderzocht. **Hoofdstuk 5** geeft aan welke mechanismen betrokken zijn bij cystevorming in de lever bij PCLD en ADPKD patiënten. Zij hebben een mutatie die aanleg geeft tot het ontwikkelen van cysten. Daarnaast is er een tweede mutatie aanwezig in het leverweefsel (somatische inactivatie; second hit), hetgeen leidt tot levercysten. Deze informatie leidde tot onderzoek van de epitheelcellen (cholangiocyten) die de cysterand bedekken en aanwezig zijn in het cystevocht. Microarray studies werden uitgevoerd om grote genetische veranderingen in het DNA van cholangiocyten uit cystevocht te onderzoeken. **Hoofdstuk 6** toont onder andere de identificatie van deze somatische deleties en geeft aanwijzingen voor mogelijke kandidaat genen.

In **hoofdstuk 7** wordt een unieke, grote PCLD familie beschreven waarbij whole-exome sequencing ten aanzien van twee aangedane familieleden is uitgevoerd. Deze studie leidde tot de identificatie van een mutatie op chromosoom 11q13.2 in het *low-density lipoprotein receptor-related protein 5* (*LRP5*) gen. Bij alle familieleden met PCLD werd deze mutatie gevonden. Screenen van het *LRP5* gen in PCLD patiënten leidde tot de ontdekking van drie nieuwe mutaties in drie verschillende families. Weefselonderzoek toonde aan dat het gemuteerde LRP5 eiwit aanwezig is in epitheelcellen van levercysten. Functioneel onderzoek wees uit dat het LRP5 eiwit minder functioneel is en leidde tot een verminderde geactiveerde Wnt cascade.

Eveneens is onderzocht of ADPKD patiënten mutaties in het *LRP5* gen hebben. Sanger sequencing toonde één nieuwe en drie bekende *LRP5* varianten waarvan wordt voorspeld dat drie varianten een rol hebben bij het ontwikkelen van cysten. **Hoofdstuk 8** beschrijft het functioneel onderzoek waarbij eveneens een verminderde geactiveerde Wnt signaleringsroute wordt gevonden.

Concluderend, genetische varianten kunnen de functie van een eiwit functioneel of structureel veranderen. Daarnaast zou een mutatie impact kunnen hebben op andere genen of eiwitten die betrokken zijn in dezelfde route. Met betrekking tot PCLD leiden de geïdentificeerde *LRP5* varianten hoogstwaarschijnlijk tot een verstoord evenwicht tussen canonical en non-canonical Wnt signaleringsroute. Deze Wnt cascade is betrokken bij processen van celgroei en celontwikkeling. Een ontregelde signalering leidt tot de vorming en de groei van lever- en niercysten.

Concluderend, mijn proefschrift geeft aan dat het in kaart brengen van het klinisch beeld (fenotype) van PCLD belangrijk is. Ten tweede, PCLD is meer genetisch heterogeen ten opzichte van eerder werd aangenomen. Tenslotte, het *LRP5* gen is een nieuw PCLD gen.

ADDENDUM

STELLINGEN
DANKWOORD
CURRICULUM VITAE
LIST OF PUBLICATIONS
ABSTRACTS AND CONFERENCES
ABBREVIATIONS

STELLINGEN

Stellingen behorend tot het proefschrift

Novel Genetic Approaches in Polycystic Liver Disease

1. Het fenotype van autosomaal dominante polycysteuze leverziekte is heterogeen.
2. Levercysten zijn dikwijls miskend en onder-gediagnosticeerd in de klinische praktijk.
3. DNA veranderingen in het lever- en nierweefsel zijn een essentieel mechanisme in het ontstaan van de ziektebeelden PCLD en ADPKD.
4. Identificatie van vele deleties in het DNA van cyste-epitheelcellen waaronder bekende tumorgenen wijzen op overeenkomstige eigenschappen betreffende tumorgenese.
5. Unieke mutaties in het *LRP5* gen zijn geassocieerd met diverse botafwijkingen, oogziekten en het ontstaan van levercysten.
6. Er zijn meer onbekende genen verantwoordelijk voor het ontwikkelen van autosomaal dominante polycysteuze levers.
7. ADPKD en PCLD patiënten hebben op klinisch vlak meer overeenkomsten dan genetisch.
8. Labwork is teamwork.
9. The energy of the mind is the essence of life. (*Artistoteles)*
10. Het leven is zo kort, men kan het niet wel genoeg verleven. (*Guido Gezelle, Vlaams dichter 1830-1899*)
11. Wa't himsels weismyt, wurdt fan in oar net opkrigen. *(Sytse ten Hoeve, Fries historicus en schrijver)*
12. It ein fan eltse reis is it paad werom. *(Piter Wilkens, Friese troubadour)*
13. Finis coronat opus.

DANKWOORD

Wetenschappelijk onderzoek is mogelijk door deelname van patiënten en hun familieleden, inspanningen van collega´s en samenwerkingsverbanden met diverse disciplines. Op deze plaats wil ik dank zeggen aan alle **patiënten**, de PCLD en ADPKD **families** die vrijwillig hebben deelgenomen aan diverse klinische en genetische onderzoeken. Dit is een belangrijke bijdrage geweest en vormt de basis van dit proefschrift. Daarnaast zijn er een aantal personen in het bijzonder die ik wil dankzeggen.

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Dr. Alexander Hoischen, beste Alex. Jouw inzet is eveneens van grote waarde geweest bij het tot stand komen van diverse projecten en met name de laatste projecten in de afrondende fase. Het verwerven en verbeteren van de kennis over nieuwe sequencing mogelijkheden binnen de genetica heeft geleid tot meer inzicht. Dank voor de vlotte samenwerking, het delen van kennis en jouw kritische blik.

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Thank you very much for the discussions, teaching, jokes and fun retreats. I appreciate the collaboration with you all and envy your strong drive to contribute to a *"significant impact on health care"*. In addition, I learned many aspects in genetics thanks to you. This motivated me and brought this research topic to the next level.

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From this position, I would like to acknowledge several **national and international societies**. Thanks to their organization of congresses and courses, I was able to present our work, to engage with science and to meet colleague young investigators. They provided young investigator bursaries and awarded dedicated and original work. Therefore, I would like to thank the Dutch societies: Nederlandse Vereniging voor Gastroenterologie (**NVGE**), Nederlandse Vereniging voor Hepatologie (**NVH**) and the **Genetica Retraite**. I also enjoyed very much the schools, masterclass and congresses of the European Association for the Study of the Liver (**EASL**), the summer school and congress weeks of the United European Gastroenterology (**UEG**). The congress of the European Society for Human Genetics (**ESHG**) was a great experience.

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Dixit, Wybrich Cnossen
Utrecht, augustus 2015

CURRICULUM VITAE

Naam: Wybrich Riemke Cnossen
Woonplaats: Utrecht
Geboortedatum: 26-05-1985

Wybrich Cnossen is geboren en opgegroeid in Sneek. Ze bezocht het Christelijk Scholengemeenschap Bogerman te Sneek en behaalde in 2003 het Gymnasium diploma (profiel Natuur en Gezondheid, fysica 1,2 en chemie 1,2).

Zij studeerde eerste kandidatuur biomedische wetenschappen aan de Universiteit Antwerpen (UA). Vervolgens startte zij in het tweede jaar geneeskunde aan de UA en voltooide deze opleiding in 2010. Gedurende deze periode zetelde zij in diverse studentenraden van de UA. Zij was hoofdredactrice van de UA Snelkrant, vice-voorzitter en later voorzitter (praeses) van de overkoepelende studentenvereniging A.S.K.-Stuwer vzw en verantwoordelijk voor de organisatie van diverse studentgerichte activiteiten op de campussen en de studentenvertegenwoordiging.

Per oktober 2010 werkte zij als junior onderzoeker aan een uitdagend project onder supervisie van Prof. dr. Joost P.H. Drenth en Prof. dr. ir. Joris A. Veltman. Dit onderzoek heeft geleid tot het huidige proefschrift. De identificatie van een nieuw gen geassocieerd met PCLD leidde tot diverse presentaties en prijzen.

Op 1 juli 2014 startte Wybrich met de opleiding tot maag-, darm- en leverarts waarbij de vooropleiding interne geneeskunde wordt gevolgd in het Rijnstate ziekenhuis te Arnhem (opleider dr. Louis Reichert).

LIST OF PUBLICATIONS

Wills ES*, **Cnossen WR***, Hoischen A, te Morsche RHM, Salomon J, Steehouwer M, de Wilt JH, Clevers H, Huch M, Hehir-Kwa J, Pfundt R, Roepman R[§], Veltman JA[§], Drenth JPH[§]. Presence of multiple infrequent germline and somatic chromosomal abnormalities underlie liver cyst development. *In preparation,*Shared first authorship, [§]Shared authorship*

Cnossen WR, Salomon J, te Morsche RHM, Koolen D, Drenth JPH. High clinical heterogeneity in isolated polycystic liver disease families. *Submitted*

Cnossen WR, Maurits JSM, Salomon J, te Morsche RHM, Waanders E, Drenth JPH. Severe polycystic liver disease is not caused by *PRKCSH* deletions. *Journal of Clinical Laboratory Analysis, **2015** September 13.*

Cnossen WR, te Morsche RHM, Hoischen A, Gilissen C, Venselaar H, Mehdi S, Bergmann C, Breuning M, Losekoot M, Peters DJM, Veltman JA, Drenth JPH. *LRP5* may contribute to ADPKD. *European Journal of Human Genetics, **2015** April 29.*

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Wolf M, **Cnossen W**, Rutsaert R, Kersschot I. *Chronic total occlusion of the left main coronary artery in a 31-year-old man. Tijdschrift voor Geneeskunde*, ***2011** February 67(3):141-145.*

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Cnossen WR, te Morsche RHM, Hoischen A, Gilissen C, Chrispijn M, Venselaar H, Mehdi S, Bergmann C, Veltman JA, Drenth JPH. Whole-exome sequencing reveals *LRP5* mutations and canonical Wnt signaling associated with hepatic cystogenesis.

Nomination for a battle, oral presentation, at the annual meeting of the Dutch Society for Gastroenterology (NVGE), March 19, **2015**. Veldhoven, The Netherlands.
NVH Young Hepatologist Award 2014

Cnossen WR, Hoischen A, Woestenenk R, Steehouwer M, Leenders M, te Morsche RHM, Veltman JA, Drenth JPH. Somatic loss of alleles from hepatic cyst epithelium allow identification of candidate genes in polycystic liver disease.
Oral presentation, symposium session, at the 22th United European Gastroenterology Week (UEGW), October 18-22, **2014**. Vienna, Austria.

Young investigators bursary
Published in supplemental file of the United European Gastroenterology Journal

Cnossen WR, te Morsche RHM, Hoischen A, Gilissen C, Venselaar H, Mehdi S, Bergmann C, Breuning M, Losekoot M, Peters DJM, Veltman JA, Drenth JPH. *LRP5* variants associated with development of polycystic kidney and liver disease.

Oral presentation at the European Society for Human Genetics (ESHG), May 30th - June 3rd, **2014**. Milan, Italy.
Award nomination

Cnossen WR, Salomon J, te Morsche RHM, Drenth JPH. Clinical heterogeneity in polycystic liver disease families.
Poster presentation at the 49th annual meeting, the International Liver Congress of the European Association for the Study of the Liver (ILC EASL), April 9-13, **2014**. London, United Kingdom.

Top 10% abstract and full bursary
Published in supplemental file of the Journal of Hepatology

Oral presentation at the annual meeting of the NVGE, October 3-4, **2013**. Veldhoven, The Netherlands.

Cnossen WR, te Morsche RHM, Hoischen A, Gilissen C, Chrispijn M, Venselaar H, Mehdi S, Bergmann C, Veltman JA, Drenth JPH. Whole-exome sequencing identifies *LRP5* variants in polycystic liver disease.

Selected for oral presentation at the 1st Masterclass dedicated to 'Hot topics in hepatology' of the EASL, November 14-16, **2013**. Bordeaux, France.

Oral presentation at the 21th UEGW, October 14-16, **2013**. Berlin, Germany.
'Oral Free Paper Prize' of the Free Paper Session: Genetics and pathogenesis.

Young investigators bursary
Published in supplemental file of the United European Gastroenterology Journal

Oral presentation at the 1st Clinical PhD retreat, Radboud university medical center, Wageningen, The Netherlands, February, **2013**.

Cnossen WR, te Morsche RHM, Hoischen A, Gilissen C, Chrispijn M, Venselaar H, Mehdi S, Bergmann C, Veltman JA, Drenth JPH. Identification of a novel gene associated with polycystic liver and kidney disease.
Poster presentation at the 48th annual ILC EASL, April 24-28, **2013**. Amsterdam, The Netherlands.
Top 10% abstract and full bursary
Published in supplemental file of the Journal of Hepatology

Poster presentation at the annual meeting of the NVGE, March 21-22, **2013**. Veldhoven, The Netherlands.

Oral presentation at the Genetica Retraite, March 21-22, **2013**. Kerkrade, The Netherlands.
First speaker's prize, 'Rolduc Award'

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Full bursary
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Cnossen WR*, Lenders E*, Van de Velde A, Berneman Z, Gadisseur A. Early detection of invasive pulmonary aspergillosis in neutropenic patients: value of the galactomannan test and high resolution computer tomography scan.
Poster presentation at the XXXIII World congress of the International Society of Hematology, October 10-13, **2010**. Jerusalem, Israel.
**shared first authorship*

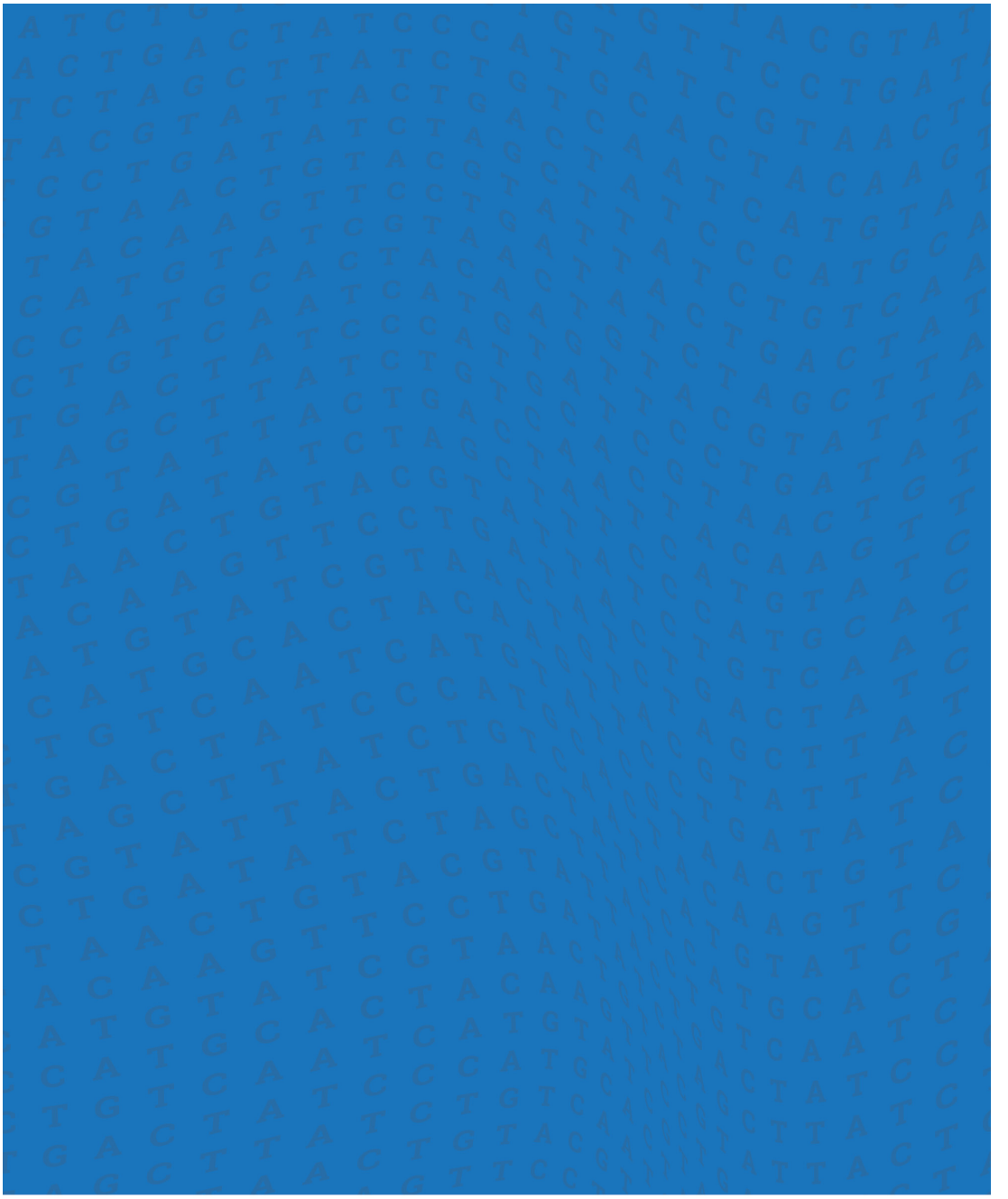
Attendance of meetings and courses

UEG Research Course: Young Investigators Meeting, UEG, October 13, **2013**. Berlin, Germany.
Abdominal ultrasound in clinical practice, Dutch Liver Week, June 12, **2012**, Rotterdam.
Nijmegen Centre for Molecular Life Sciences (NCMLS) PhD retreat, Wageningen. May **2011** and **2012**, poster presentations.
47th annual meeting, the ILC EASL, April 18-22, **2012**. Barcelona, Spain.
Annual meeting of the NVGE, March 17-18, **2011**. Veldhoven.
ASNEMGE/EAGE Summer School of Gastroenterology, June 16-19, **2011**. Prague, Czech Republic.
46th annual meeting, the ILC EASL, March 30-April 4, **2011**. Berlin, Germany.
Monothematic Conference 'Basic liver course' of the EASL, February **2011**. Groningen.

ABBREVIATIONS

AAA	abdominal aorta aneurysm
ADPKD	autosomal dominant polycystic kidney disease
ADPLD	autosomal dominant polycystic liver disease
AE2	anion exchanger 2
ARPKD	autosomal recessive polycystic kidney disease
AQP1	aquaporin 1
cAMP	adenosine 3', 5'-cyclic monophosphate
CD	Caroli disease
CFTR	cystic fibrosis transmembrane conductance regulator
CHF	congenital hepatic fibrosis
CNA	copy number aberration
CNV	copy number variation
CS	Caroli syndrome
CT	computer tomography
DKK3	dickkopf Wnt signaling pathway inhibitor 3
DPM	ductal plate malformation
DQ	dosage quotient
ELAVL3	embryonic lethal abnormal vision drosophila homolog-like 3
Epac	exchange protein
ER	endoplasmic reticulum
ERK	extracellularly regulated kinase
ESRD	end stage renal disease
FACS	fluorescent-activated cell sorting
HNF1ß	hepatocyte nuclear factor-1-beta
HVOO	hepatic venous outflow obstruction
ICA	intracranial aneurysm
IVC	inferior vena cava
LOH	loss of heterozygosity
LPO	left probe oligonucleotide
LRP5	low density lipoprotein receptor-related protein 5
LVH	left ventricle hypertrophy
MAPK	mitogen-activated protein kinase
MELD	model for end-stage liver disease
MLPA	multiplex ligation-dependent probe amplification
MRI	magnetic resonance imaging
ORF	open reading frame
PC1, PC2	polycystin-1, -2
PCLD	autosomal dominant polycystic liver disease
PCP	planar cell polarity
PCR	polymerase chain reaction
PKA	protein kinase A

PKD1, PKD2	polycystic kidney disease-1, -2
PLD	polycystic liver diseases
PKD1	polycystic kidney disease 1
PKD2	polycystic kidney disease 2
PKDTS	TSC2/PKD1 contiguous syndrome
PKHD1	polycystic kidney and hepatic disease 1
PRKCSH	protein kinase C substrate 80K-H (80 kDa protein, heavy chain)
RPO	right probe oligonucleotide
SEC63	Saccharomyces cerevisiae homolog 63
SNP	single nucleotide polymorphism
SR	secretin receptor
SSTR	somatostatin receptor
TSC	tuberous sclerosis complex
VMC	von Meyenburg complexes
Wnt	wingless-type MMTV integration site family member
WT	wild type



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